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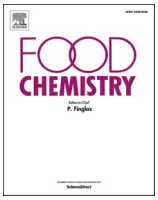

Comparative compositions of grain of tritordeum, durum wheat and bread wheat grown in multi-environment trials

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ABSTRACT

Three genotypes each of bread wheat, durum wheat and tritordeum were grown in randomized replicated field trials in Andalusia (Spain) for two years and wholemeal flours analysed for a range of components to identify differences in composition. The contents of all components that were determined varied widely between grain samples of the individual species and in most cases also overlapped between the three species. Nevertheless, statistically significant differences between the compositions of the three species were observed. Notably, tritordeum had significantly higher contents of protein, some minerals (magnesium and iron), total phenolics and methyl donors. Tritordeum also had higher levels of total amino acids (but not asparagine) and total sugars, including raffinose. By contrast, bread wheat and tritordeum had similar contents of the two major dietary fibre components in white flour, arabinoxylan and β-glucan, with significantly lower contents in durum wheat.

1. Introduction

Tritordeum is a novel hexaploid amphiploid cereal species with the genome constitution AABBH^{ch}H^{ch} which was generated in the 1970s by combining the genomes of tetraploid durum wheat (*Triticum turgidum*, var. *durum*) and the perennial wild barley species *Hordeum chilense* (Martin et al. 1999). Tritordeum is adapted to the same Mediterranean environments as durum wheat but has rheological and baking performances similar to those of bread wheat (Martin et al., 1999) with good organoleptic properties and high consumer acceptability (Vaquero et al., 2017; Sánchez-León et al., 2020). It is typically grown as a winter-sown crop and is most competitive under Mediterranean-type conditions of relatively warm winters with rainfall in Autumn and Spring as it makes strong vegetative growth during the winter months. Much of the original selection of tritordeum breeding lines was made in Córdoba in Andalusia (Martin et al. 1999) and it shows tolerance to drought, heat and salinity stress, maintaining higher biomass production under stress conditions (Yousfi et al. 2010). Tritordeum has good general agronomic performance (Kakabouki et al., 2020), is suitable for cultivation in conventional and organic agriculture (Visioli et al., 2020) and is reported to have a lower environmental impact with higher nitrogen uptake (and hence a lower requirement for nitrogen fertilization) and higher water use efficiency than durum wheat (Martin et al., 1999). In addition to suitability for breadmaking, malted tritordeum grain has high enzymatic activity and is suitable for the production of quality beers, either from admixtures with barley malt or from 100% tritordeum malt (Zdaniewicz et al., 2020; Yding et al., 2022).

In recent years it has been suggested that older types of wheat

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(sometimes called heritage or heirloom varieties) and "ancient" wheats (einkorn, emmer and spelt) offer health benefits over modern wheat varieties. In this regard, tritordeum has attracted considerable interest as an alternative cereal with putative environmental and health benefits. For example, it has been reported to have higher contents of a range of phytochemicals (carotenoids, xanthophylls, phenolic acids) (Atienza et al., 2007; Giordano et al., 2019; Suchowilska et al., 2021). Furthermore, it has been suggested to have a lower content of indigestible gliadin peptides and amylase trypsin inhibitors which may induce inflammation and intestinal barrier disfunction, and therefore to be more suitable for individuals suffering from adverse reactions to wheat (Vaquero et al., 2017; Sánchez-León et al., 2020; Haro et al., 2022; Nitride et al., 2022; Russo et al., 2022).

However, only a few comparative studies of the grain compositions of tritordeum and wheat have been reported and further work is required to establish the extent of variation in composition between genotypes and grain samples grown under different conditions. We therefore selected three genotypes each of tritordeum (Aucan, Bulel and HT444 [a breeding line in the process of registration]), durum wheat (Iride, Avispar and Amilcar) and bread wheat (Gazul, Capo and Artur Nick), all of which are adapted to Mediterranean growth conditions in Spain, and compared their compositions when grown in replicated plots on two sites for two years.

2. Materials and methods

2.1. Grain samples

Three genotypes each of tritordeum (Bulel, HT444, Aucan), durum wheat (Iride, Avispar, Amilcar) and bread wheat (Gazul, Capo, Artur Nick) were selected. All are commercial cultivars except the tritordeum line HT444 which is an advanced breeding line which is undergoing registration as a cultivar. They are therefore all described as "cultivars" in the following text. All were grown at two locations in Andalusia, Spain (Santaella: 37° 55′ 38.50″ N, −4° 84′ 59.40″ W and L'Algamarilla: 37° 78′ 31.30″ N-5° 04′ 30.40″ W) in 2017–2018 and 2018–2019 in three replicate randomized plots of 14 square meters. 150 kg/Ha of nitrogen was applied in two treatments. Sowing dates were between 14 and 28 November (2018) and 18 November and 3 December (2019) and harvests between 26 and 29 June (2018) and 15 and 25 June (2019). Grain samples from individual plots were milled in two stages, firstly, a Retsch ZM 200 Model Ultra-Centrifugal Mill (Retsch Gmbh, Dusseldorf, Germany) using a 0.5 mm ring sieve and then a Glen Creston Ball Mill (Retsch Gmbh, Dusseldorf, Germany) using 5 ball bearings in a 5 cm diameter cannister for 4 min for each sample.

2.2. Genotyping

The Axiom 35k Wheat Breeders Genotyping Array (Thermo Fisher Scientific, Inc., Waltham, MA) (comprising 35,143 SNP markers) was used to genotype the 13 samples using the Affymetrix GeneTitan (Thermo Fisher Scientific, Inc.) system according to the procedure described by Affymetrix (Life Technologies, 2017). Allele calling was performed using the Affymetrix proprietary software package Axiom Analysis Suite and prior model 'Axiom_WhtBrd-1.r3′. A reduced Dish QC threshold of 0.8 and call rate cut-off of 90% was used to adjust for the lower call rates typically obtained from hybridizing wheat relatives to the array. A distance matrix was generated from the genotype scores using R package SNPRelate (Bioconductor Open Source, Harvard, MA, USA) (Zheng et al., 2012) The 'exact' algorithm was used for calculations and data processed without MAF filtering or Bayesian normalization. The first two eigenvalues accounting for over 30% of the variance (EV1: 25.55%; EV2: 5.53%) were plotted as a PCA plot.

2.3. Mineral analysis

Samples of biological replicates were oven-dried at 80° C overnight, weighed and digested using a mixture of nitric and perchloric acids (85:15 v/v) in open tube digestion blocks, followed by a programmed heating digestion: 60° C for 180 min, 100° C for 60 min, 120° C for 60 min, 175° C for 90 min and 50° C until dry. The acids were removed by volatilisation and the residues dissolved in nitric acid (5% v/v). Minerals were determined with an Optima 7300 DV Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) (Perkin Elmer, Waltham, MA, USA). The analyses were monitored using certified external standards and in-house standard materials. Standards and check samples were monitored and recorded using Shewhart Control Graphs and computer-based quality control packages.

Total nitrogen was determined using the American Society for Testing and Materials (ASTM) standard protocol E1019 using a Leco Combustion analysis system (Leco Corp., St Paul, MN, USA) based on the Dumas method. Total nitrogen was converted to protein content using the factor Nx5.7.

2.4. Determination of total phenolics

Total phenolics were determined based on Gao et al. (2002). Briefly, three technical replicates (75 mg) of each biological replicate were vortexed with 1.5 ml acidified methanol and then mixed at 850 rpm on an Eppendorf Thermomixer (Eppendorf Ltd., Stevenage, UK) for 2 h at 23° C. After centrifugation (Eppendorf Ltd., Stevenage, UK) at 5,000×*g* for 10 min, 1 ml of supernatant was removed into a fresh tube and 200 μl aliquots mixed with 1.5 ml of x10 diluted Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, MO, USA) and left to stand for 5 min. 1.5 ml of 6% (w/v) aqueous sodium carbonate solution was then added, mixed and stood at room temp for 90 min. The absorbance at 725 nm was then measured (Jenway 6715 UV/Vis spectrophotometer, Cole-Parmer, St Neots, UK) and the concentration of phenolics calculated using ferulic acid (Sigma-Aldritch) as a standard.

2.5. Determination of polar metabolites by ^1H NMR spectroscopy

30 mg samples of biological replicates were extracted at 50 °C using D_2O:CD_3OD (80:20) containing 0.05% d_4– trimethylsilylpropionate (TSP) (1 ml) as internal standard (Shewry et al., 2017). ^1H NMR spectra were acquired at 300 °K using an Avance Neo Spectrometer (Bruker Biospin, Coventry, UK) operating at 600.0528 MHz, with a cryoplatform and a 5 mm triple resonance inverse probe. Spectra were acquired using 16 scans of 65,536 data points with a spectral width of 7143 Hz. A water suppression pulse sequence (zgpr) was used with a 90° pulse and a 5 s relaxation delay. Spectra were Fourier-transformed using an exponential window with line broadening of 0.5 Hz. Phasing and baseline correction were carried out automatically and spectra were reduced using Amix (Analysis of MIXtures software, BrukerBiospin) to ASCII files containing integrated regions of equal width (0.01 ppm) and spectral intensities scaled to the d_4-TSP region (δ0.05 to –0.05). Signal intensities for spectral regions for major metabolites were extracted by comparison to spectra of known standards run under the same conditions.

2.6. Determination of arabinoxylan and β-glucan by enzyme fingerprinting

Three technical replicates of each biological replicate were digested using a mixture of endoxylanase and lichenase (β-glucanase) to release arabinoxylan oligosaccharides (AXOS) and *gluco*-oligosaccharides (GOS) comprising 3 and 4 residues (G3, G4), respectively. These were separated by high performance anion-exchange chromatography (HP-AEC) using a Dionex Carbopac PA-1 column (Thermo-Fisher Scientific Inc., Waltham, MS, USA) with dimensions 2 mm × 250 mm and a flow rate of 0.25 ml/minutes as described (Lovegrove et al., 2013; https://doi.org/1

0.17504/protocols.io.babriam6). The areas under the AXOS peaks and under the G3 and G4 GOS peaks were combined to give total AX and total β-glucan (expressed in arbitrary units), respectively.

2.7. Statistical analysis

All data were analysed using analysis of variance (ANOVA) in Genstat 21 (VSN International, Hemel Hempstead, UK). The four trials (2 sites × 2 years) were treated as blocks for analysis and a completely randomized allocation of genotypes to plots within each trial was assumed. The treatment structure, Grain/(genotypeBread + genotypeDurum + genotypeTritordeum), was used to allow a higher level comparison between the three grain types and also more detailed comparisons between the genotypes within each of the three grain types. Some variables were transformed in order to meet the normality and homoscedasticity assumptions of the analysis. These transformations are indicated in the tables of means and p values.

Principal Component Analysis (PCA) was performed in SIMCA-P software (version 13, Sartorius UK Ltd, Epsom, UK).. Boxplots were created in R (Version 4.0.2) using the ggplot2 package.

3. Results

Three cultivars each of tritordeum (Bulel, HT444, Aucan), durum wheat (Iride, Avispar, Amilcar) and bread wheat (Gazul, Bologna and Artur Nick) were selected for comparison. The cultivars were grown in replicate field trials for two years (2018, 2019) on two sites in Spain. Because only two sites and years were compared it was not possible to calculate the separate contributions of genotype, environment and G × E interactions to the variation in composition. The four site × year combinations were therefore treated as "environments" in the statistical analyses.

The means and ranges of contents of components in the three species are shown in Figures and the means, standard errors of means (SEMs) and p-values from ANOVA analyses in Supporting Material Tables S1 to S4. The p-values from Anova analyses of selected components are also given in Table 1.

Means and SEMs for components in the cultivars of the three species are given in Supporting Material Tables S5 and S6.

3.1. Genomic relationships

Bread wheat is a hexaploid species with the genome constitution AABBDD while durum wheat is a tetraploid with the AABB genomes only. Tritordeum is a new species generated from crossing durum wheat with *Hordeum chilense* and therefore has the AABB genomes combined with the $H^{ch}H^{ch}$ genome. The broad genetic relationships between the A and B genomes in the cultivars and species were determined using the Axiom 35k Wheat Breeders Genotyping Array, comprising 35,143 SNP markers, and the data analysed by principal component analysis (Fig. 1). The PCA analysis showed clear separation of the bread and durum wheats, with the latter forming a very tight cluster while the bread wheats were widely separated. The tritordeum lines also form a tight cluster close to, but separate from, the durum wheat cultivars. This is consistent with the shared A and B genomes and absence of the D genome in tritordeum and durum wheat and also indicates divergence between the A and B genomes present in these species and those in bread wheat. The Axiom Wheat HD genotyping array is designed for bread wheat and hence would not be appropriate for the H^{ch} genome of tritordeum.

3.2. Grain weight and contents of protein and minerals

The thousand grain weights (TGW) of the three cereal species, determined in 2019 only, are shown in Fig. 2A. Whereas tritordeum and bread wheat had similar mean values, the range was much wider for bread wheat. Durum had a significantly higher TGW.

Table 1
P-values from ANOVA of TGW, nitrogen, minerals, phenolics, metabolites and proportions, ratios and structures of oligosaccharides derived from arabinoxylan (AXOS) and β-glucan (GOS) in grain of three cereal species grown on two sites in 2018 and 2019.

	Grain	Grain. genotype bread wheat	Grain. genotype durum	Grain. genotype tritordeum
log(TGW) (2019 only)	**<0.001**	**<0.001**	0.362	**0.043**
%N	**<0.001**	**0.002**	0.677	**<0.001**
sqrt(Ca)	0.376	**<0.001**	0.931	0.99
sqrt(Fe)	**<0.001**	0.082	0.531	**<0.001**
Mg	**<0.001**	**0.039**	**0.024**	0.86
sqrt(Zn)	**<0.001**	**0.031**	0.162	0.132
total phenolics	**<0.001**	**0.003**	0.674	0.337
sqrt(raffinose)	**<0.001**	**<0.001**	0.557	**<0.001**
asparagine	**<0.001**	**<0.001**	0.383	**<0.001**
sqrt(glycine betaine)	**<0.001**	**<0.001**	0.466	**<0.001**
choline	**<0.001**	0.287	0.736	0.411
log(galactinol)	**<0.001**	**<0.001**	0.359	**<0.001**
log(inositol)	**<0.001**	**<0.001**	0.876	0.112
total amino acids	**<0.001**	0.094	0.783	**<0.001**
log(total organic acid)	**<0.001**	**<0.001**	0.545	**<0.001**
sqrt(total methyl donors)	**<0.001**	**<0.001**	0.507	**<0.001**
sqrt(total sugars)	**<0.001**	**<0.001**	0.16	**0.008**
$\log_e$ (TOT-AXOS)	**<0.001**	**<0.001**	0.591	**0.023**
$\log_e$ (TOT-BG)	**<0.001**	**0.006**	**0.04**	**<0.001**
$\log_e$ (Ratio G3:G4 GOS)	0.657	**<0.001**	0.097	0.408
$\log_e$ (Ratio TOT -AXOS: TOT-BG)	**<0.001**	**0.001**	**0.007**	**0.022**
sqrt(Ratio M:D AXOS)	**<0.001**	**<0.001**	0.523	**<0.001**

Statistically significant values ($p < 0.05$) are given in bold. Some variables required transformation, square root (sqrt) or $\log_e$ to meet the assumptions of the analysis.

Grain protein (determined as N × 5.7), magnesium, zinc and iron were all significantly higher in tritordeum than in bread and durum wheats (Fig. 2B, D-F), but there were no significant differences between the three species in grain calcium (Fig. 2C, Table 1 and Supporting Material Table S1). There were significant differences between cultivars of bread wheat for all minerals except for iron. Magnesium was significantly different between cultivars of durum wheat and iron was significantly different between cultivars of tritordeum (Table 1).

3.3. Contents of total phenolics and polar metabolites

Phenolics are the major phytochemicals in wheat grain and total phenolics were therefore determined in the wholemeal flours. The contents in the three species overlapped but were significantly higher in tritordeum with significant differences between cultivars for bread wheat only (Fig. 2G, Table 1, Supporting Material Table S1).

^{1}H NMR spectroscopy was used to determine polar metabolites extracted in methanol/water. This quantified thirteen free amino acids, monosaccharide (glucose, fructose, galactose) and disaccharide (maltose, sucrose) sugars and organic acids (malic, acetic, fumaric). It also quantified raffinose (a trisaccharide sugar) and two biosynthetic precursors of raffinose, the sugar alcohols inositol and galactinol, and the "methyl donors" choline and betaine. Selected components and groups of components are presented in Fig. 2H-Q and Table 1 and the full analyses in Supporting Material Tables S1 and S2.

The content of total free amino acids (alanine, aspartic acid, asparagine, glycine, glutamic acid, glutamine, γ-amino butyric acid (GABA), isoleucine, leucine, phenylalanine, tyrosine, tryptophan and valine) was

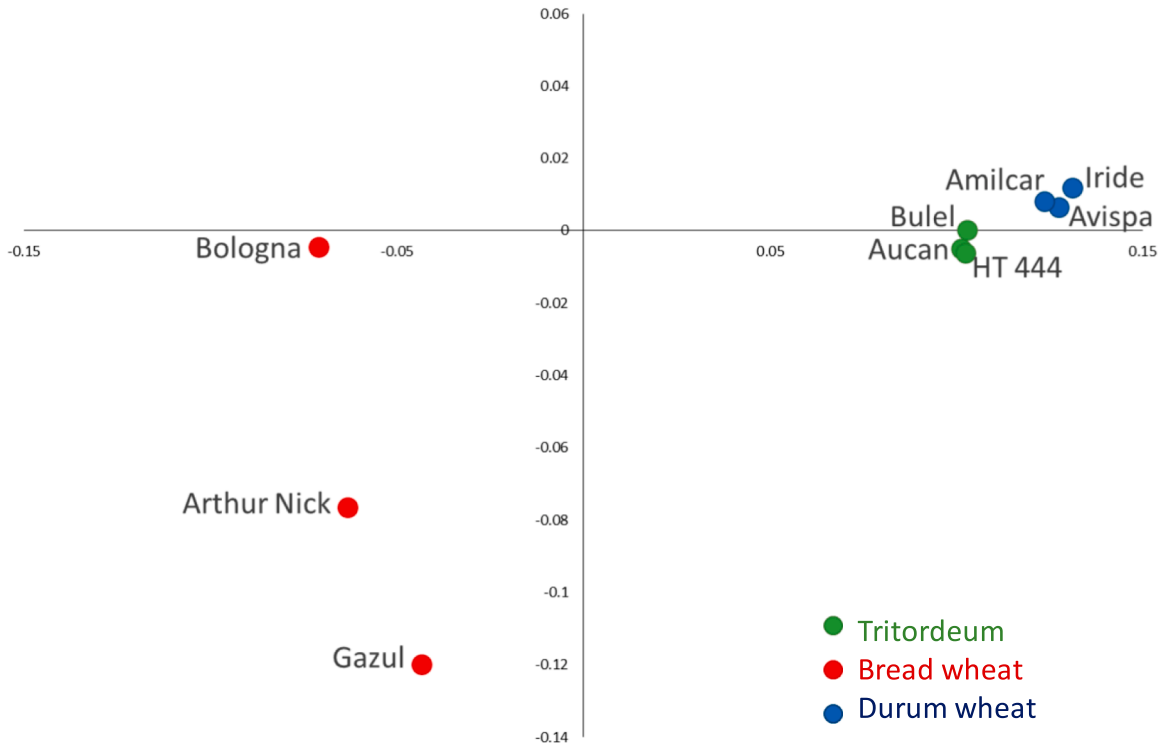

Fig. 1. Genomic relationships of the 9 cultivars, illustrated by Principal Component Analysis of markers determined using the Axiom HD Genotyping Array (comprising 819,571 SNP markers).

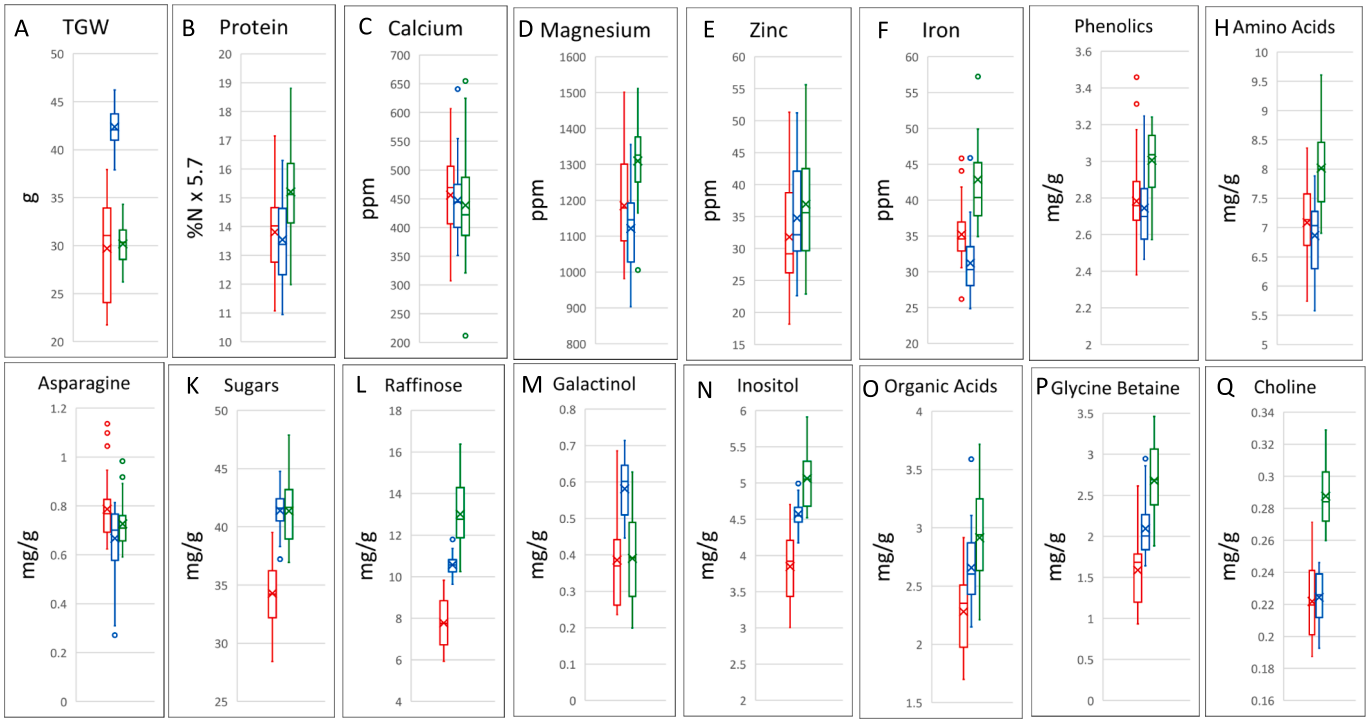

Fig. 2. Thousand grain weights (A) (2019 only) and contents of protein (B), minerals (C-F), total phenolics (G) and selected polar metabolites and groups of metabolites (H-Q) in grains of the three cereal species grown in four environments. Code: red, bread wheat; blue, durum wheat; green, tritordeum The bar shows the range of the whole data set. The box shows the middle two quartiles, separated by the horizontal line which is the median, and the vertical lines are the upper and lower quartiles respectively. Outliers are shown as circles. The x is the mean average.

significantly higher in tritordeum than in bread and durum wheats (Fig. 2H, Table 1, Supporting Material Table 1). By contrast, the content of the amino acid asparagine was slightly lower in tritordeum than in bread wheat but slightly higher than in durum wheat (Fig. 2J, Table 1, Supporting Material Table 1).

The contents of total sugars were similar in durum wheat and tritordeum but lower in bread wheat (Fig. 2K, Table 1) but the contents of raffinose and its precursor inositol (but not galactinol) were significantly higher in tritordeum and lower in bread wheat (Fig. 2L, M and N, Table 1, Supporting Material Table 1).

The concentrations of glycine betaine in the three cereals were about 10-fold greater than those of choline, as reported previously for wheat (Corol et al., 2012), and the contents of both components were significantly higher in tritordeum than in bread and durum wheats (Fig. 2 P and Q, Table 1, Supporting Material Table 1).

Finally, the content of total organic acids (malic, fumaric and acetic) was highest in tritordeum and lowest in bread wheat (Fig. 2O, Table 1, Supporting Material Table 1).

Significant differences in the above-mentioned metabolites were also observed between the cultivars within bread wheat and tritordeum but not within durum wheat (Supporting Material Table S2). This difference may relate to the durum cultivars being closely related, as shown by the genomic comparison in Fig. 1.

Further information on differences in the contents of individual polar

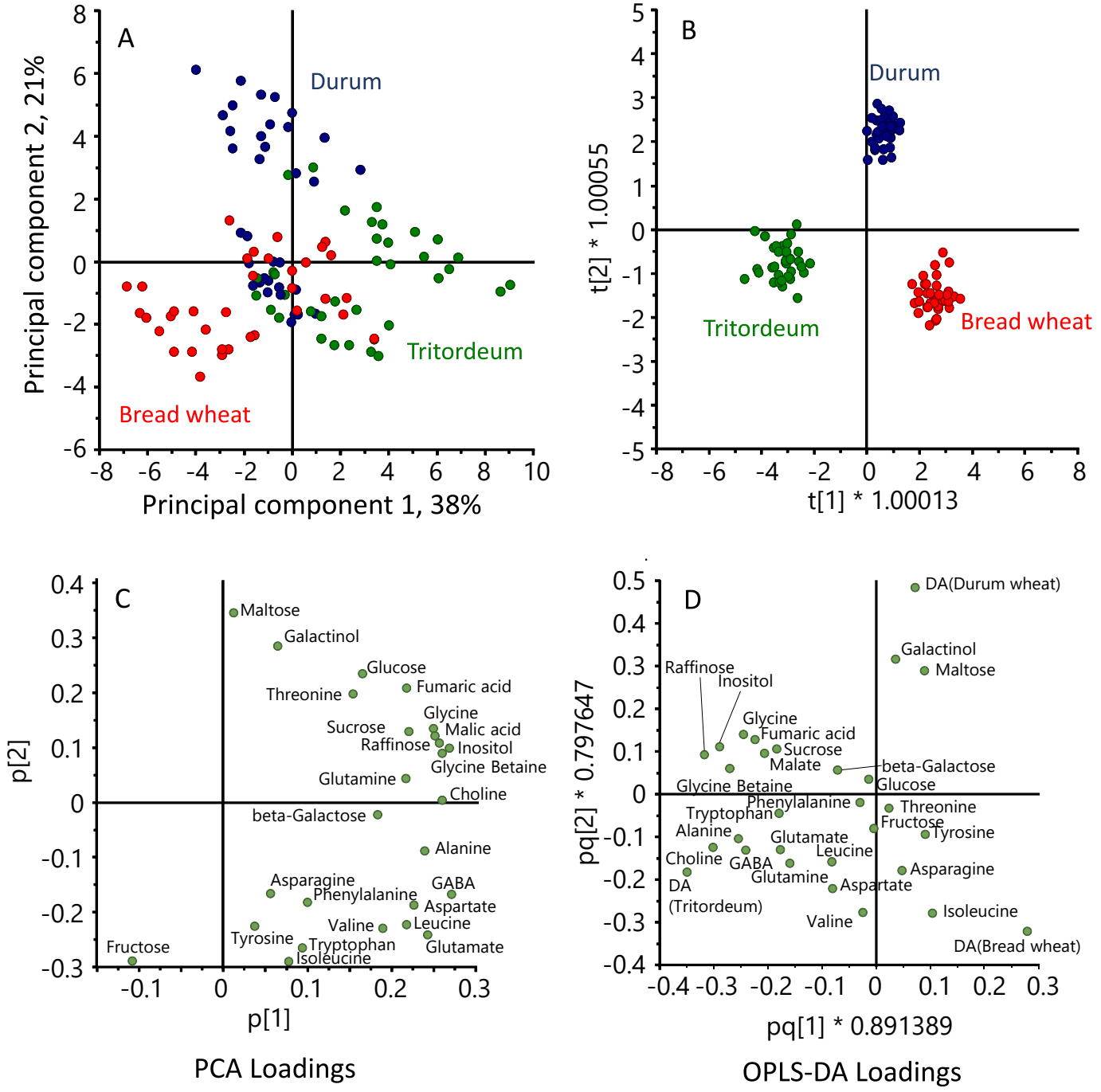

Fig. 3. Multivariate analysis of the contents of polar metabolites in grains of the three cereal species grown in four environments. A, principal component analysis (PCA); B, orthogonal partial least squares discrimination analysis (OPLS-DA) selecting for differences between cereal types. Code: red, bread wheat; blue, durum wheat; green, tritordeum C, D, loadings plots for the separations in A and B, respectively.

metabolites between the three species is given by multivariate analysis (Fig. 3). Principal component analysis (PCA) gave only partial separation of the species (Fig. 3A) so supervised multivariate analysis (orthogonal partial least squares discrimination analysis, OPLS-DA) was used to select for differences between the species. This clearly separated the three species (Fig. 3B) and the loadings plot (Fig. 3D) showed that durum wheat was particularly rich in galactinol and maltose, bread wheat in the free amino acids threonine, tyrosine, asparagine and isoleucine and tritordeum in choline and the free amino acids alanine, GABA, glutamate and aspartate. These differences are also shown by difference plots based on the OPLS-DA analysis (Supporting Material Fig. S1A-C).

3.4. Contents and structures of arabinoxylan and β-glucan fibre

The contents of arabinoxylan and β-glucan in wholemeal flours of the three cereal species were determined using enzyme fingerprinting. In this procedure the arabinoxylan and β-glucan polymers are digested with endoxylanase and lichenase (β-glucanase) enzymes to release oligosaccharides (arabinoxylan oligosaccharides, AXOS, and *gluco*-oligosaccharides, GOS, respectively). Both enzymes have high substrate specificity releasing AXOS and GOS which have defined structures. Hence, the proportions of the AXOS and GOS provide information on the polymer structures while their combined amounts can be used to quantify the polymer amounts. The amounts of AX and β-glucan calculated as the total areas of the AXOS and GOS peaks separated by HP-AEC are shown in Fig. 4A,B with means and SEMs in Supporting Material Table S3 and p -values from ANOVA in Table 1. The contents of both polymers were lower in durum wheat while tritordeum had a slightly higher content of arabinoxylan, which was reflected in a higher ratio of AX: β-glucan (Fig. 4C,D).

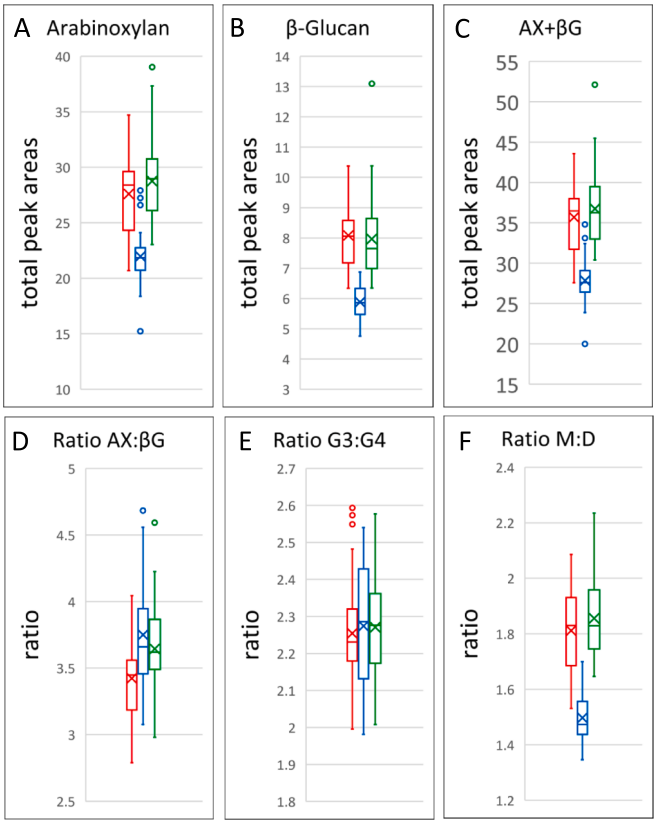

Fig. 4. Contents, ratios and structures of arabinoxylan and β-glucan in grains of the three cereal species grown in four environments. Code: red, bread wheat; blue, durum wheat; green, tritordeum The box shows the middle two quartiles, separated by the horizontal line which is the median, and the vertical lines are the upper and lower quartiles respectively. Outliers are shown as circles. The x is the mean average.

The proportions of individual AX and GOS also provide information on variation in the structures of the two polymers. AX comprises a linear chain of arabinose residues, some of which are substituted with one (monosubstituted) or two (disubstituted) arabinose residues. The pattern of substitution may affect the properties of AX, including solubility and fermentability. Similarly, β-glucan comprises linear chains of glucose molecules linked by β(1–4) bonds interspersed with β(1–3) bonds, the latter generally occurring every 3 or 4 glucose residues. The distribution of β(1–3) bonds results in bends in the linear glucan molecules which affect their solubility and viscosity. Lichenase releases mainly *gluco*-oligosaccharides (GOS) of 3 or 4 glucose residues (called G3 and G4), in proportions which reflect the relative abundances of β-(1–3) and β-(1–4) bonds.

Full datasets for the individual AXOS and GOS are given in Supporting Material Table S3 and p-values from ANOVA in Supporting Material Table S4. The ratios of monosubstituted:disubstituted AXOS calculated from these data are presented in Fig. 4F and Table 1, which shows that the ratio was significantly lower for durum wheat than for bread wheat or tritordeum. By contrast, the ratio of G3:G4 GOS did not differ significantly between the three species (Fig. 4E and Table 1). Differences in these parameters between cultivars within the three species were also observed, for total AXOS, total β-glucan and the ratios TOT-AX:TOT-β-glucan, monosubstituted:disubstituted AXOS and G3:G4 GOS in bread wheat, for all except G3:G4 GOS in tritordeum and for total β-glucan and the ratio TOT-AX:TOT-β-glucan in tritordeum (Table 1).

Multivariate analysis of the proportions of individual AXOS and GOS by PCA gave a partial separation of the three species (Fig. 5B) with a clear separation by OPLS-DA (Fig. 5B). The loading plots for the OPLS-DA analysis showed that tritordeum had higher proportions of substituted AXOS (Fig. 5D) and this was also shown by difference plots (Supporting Material Fig. S1D,E).

4. Discussion

We have compared the compositions of wholemeal flours of three cultivars each of bread wheat, tritordeum and durum wheat gown in replicate plots on two sites for two years (ie. four environments). All cultivars are adapted to the region of growth. Their compositions are therefore relevant to those of commercial grain samples gown in Southern Spain. However, because the numbers of cultivars and environments compared in the present study were small, they are unlikely to represent the full range of compositions encountered in commercial samples.

The concentrations of bioactive compounds in cereal grains are often inversely correlated with the thousand grain weight, as larger grains have higher contents of starch which dilutes other grain components (see, for example, Ward et al., 2008). In the present study there was little difference between the TGW of bread wheat and tritordeum, so the lower contents of components in the bread wheats were clearly not due to starch dilution. However, the durum wheat grains were significantly heavier which could have resulted in dilution of some components such as fibre.

All components showed wide ranges in concentration between samples of the individual species which largely overlapped between the species. Nevertheless, statistically significant differences between the composition of the three species were observed. In particular, tritordeum had significantly higher contents of protein, some minerals (magnesium and iron), total phenolics and methyl donors. It also had higher contents of total free amino acids (but not asparagine) and total sugars, including raffinose. Some of these differences could be relevant to health.

Previous studies have shown higher contents of bound phenolic acids (the major phenolic fraction in wheat grain), flavonoids (Suchowilska et al., 2021), minerals (Suchowilska et al., 2023) and carotenoids

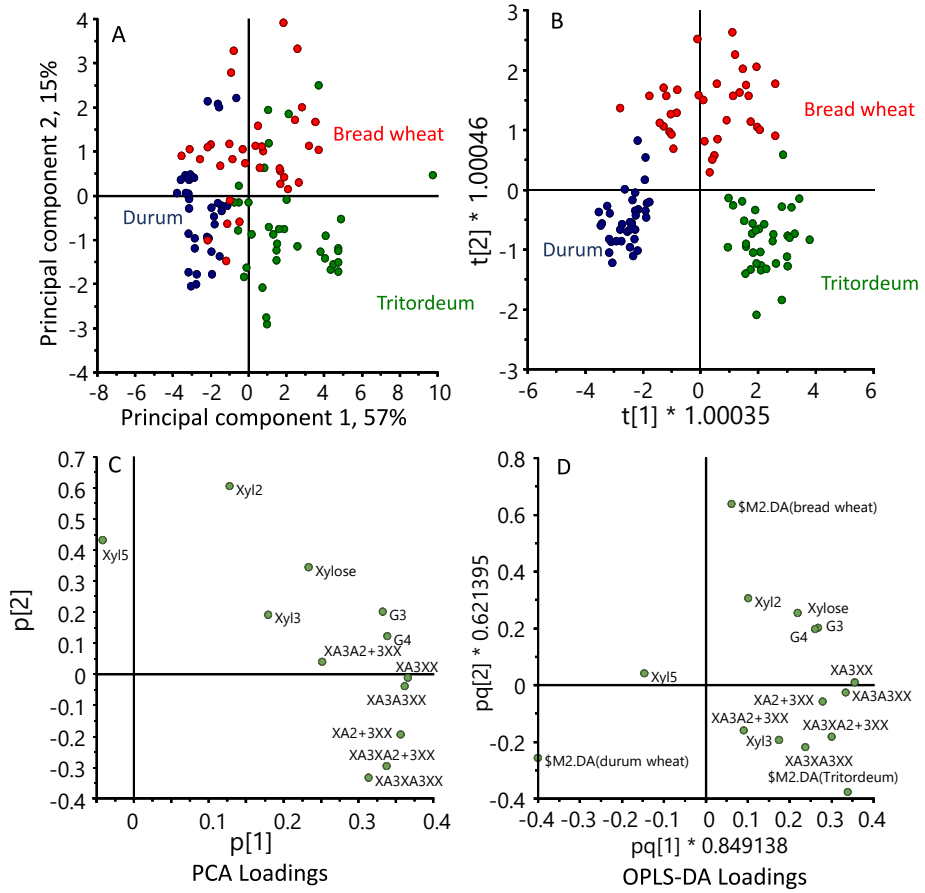

Fig. 5. Multivariate analysis of the contents of AXOS and GOS in grains of the three cereal species grown in four environments. A, principal component analysis (PCA); B, orthogonal partial least squares discrimination analysis (OPLS-DA) selecting for differences between cereal species. Code: red, bread wheat; blue, durum wheat; green, tritordeum C, D, loadings plots for the separations in A and B, respectively.

(Atienza et al., 2007) in tritordeum than in durum wheat, but no difference between the contents of alkylresorcinols (a group of phenolic lipids) between the grains of bread wheat and tritordeum (Petrazzani et al., 2021). Phenolics present in grain have been proposed to be associated with a range of health benefits, which in some cases may be related to their high antioxidant capacity (Young and Woodside, 2001; Călinoiu and Vodnar, 2018, Tian et al., 2022). However, the major phenolic compound in wheat is ferulic acid which is largely bound to arabinoxylan in the cell walls (Li et al., 2008). Although, a small proportion of bound ferulic is released during fermentation in the colon, it can be more fully released by including the enzyme feruloyl esterase during food processing, leading to improved vascular function (measured as flow mediated dilatation) in a short-term dietary intervention study (Turner et al., 2021).

The contributions of free amino acids and small sugars (mono-, di- and trisaccharides) to human nutrition and health are small as the vast majority of amino acids and sugars are present in proteins and polysaccharides (notably starch), respectively. However, several individual components do have relevance to health. The amino acid asparagine is of interest as it is a precursor of acrylamide which is formed by a Maillard reaction between asparagine and reducing sugars during grain processing (Mottram et al., 2002). Furthermore, the formation of acrylamide is correlated with the free asparagine content of wheat flour, rather than the content of reducing sugars (Muttucumaru et al., 2008). Hence, the similarity in the asparagine contents of the three species is notable.

The trisaccharide raffinose is of interest as it is not digested or absorbed in the human small intestine but is fermented in the colon which may have impacts on heath. Firstly, raffinose has prebiotic properties and fermentation leads to the release of short chain fatty acids known to mitigate intestinal inflammation and impaired barrier function and support gut associated immune competence, in favor of reduced risk of bowel disease (Alvandi et al., 2022; Venegas et al., 2019). However, because of this rapid fermentation raffinose is also classed as a FODMAP (Fermentable Oligosaccharides, Disaccharides, Monosaccharides And Polyols), a group of components known to result in discomfort in those suffering from irritable bowel syndrome (IBS). Consequently, low FODMAP diets are recommended for IBS patients (Barrett and Gibson, 2012). However, the relevance of the differences in raffinose content observed in the present study to health may be low as the major FODMAPs in grain are fructans, which account for up to 3% of the grain dry weight (i.e., 5–10 times higher than the concentration of raffinose) (Grausgruber et al., 2020). It is notable that Russo et al (2022) reported that a tritordeum-based diet resulted in a reduction of gastrointestinal symptoms in a randomized controlled pilot trial with patients suffering with IBS-diarrhea variant. They suggested that this occurs due to reductions in intestinal permeability, mucosal inflammation and dysbiosis. However, this was a pilot study with only 16 patients and comparisons with other wheat-based diets were not performed. Larger studies with appropriate controls are therefore required to draw definitive conclusions.

Glycine betaine and its biosynthetic precursor choline are beneficial for human health as they act as “methyl donors” in the homocysteine cycle, providing methyl groups for the re-methylation of homocysteine to methionine, reducing the risk of cardiovascular disease. Tritordeum had significantly higher contents of both components, with the content of glycine betaine being close to the amount required by the European Food Safety Authority (EFSA) for health claims: this is 500 mg per

portion (Tiihonen et al., 2014) which corresponds to about 200 g dry wt. of wholemeal bread.

Dietary fibre is an essential component of the human diet and cereals are a major source of fibre in the European diet (providing about 40% of fibre intake in the UK). Bread is the major cereal-based food but the fibre composition and content differ depending on whether it is made from whole grain or refined white flour. Whole-grain wheat bread contains about 11.5–15.5% dry wt. total fibre, of which about half (5.5–7.4% dry wt.) is arabinoxylan (AX), with a lower content of β-glucan (0.51–0.96% dry wt.) (Andersson et al., 2013). By contrast, white flour contains only about 4% dry wt. fibre, with AX and β-glucan accounting for about 70% and 20% of the total non-starch polysaccharides, respectively (Mares and Stone, 1973). The third major dietary fibre component in whole grain is cellulose. This is derived from the pericarp and not present in white flour.

By contrast, the major dietary fibre component in the starchy endosperm and whole grain of cultivated barley (*Hordeum vulgare* L.) is β-glucan which accounts for about 75% of the total polysaccharides in the cell walls (Fincher, 1975). β-glucans from barley and oats have established health benefits (El Khoury et al., 2012; Jayachandran et al., 2018). Consequently, there is significant interest in increasing the content of β-glucans in wheat, including the introgression of the trait from barley.

However, the contents of β-glucan in the bread wheat and tritordeum lines in the current study were similar, with a slightly higher content of AX in the tritordeum lines resulting in a higher ratio of AX:β-glucan. This is because *Hordeum chilense* is not closely related to cultivated barley, being classified in a separate section of the genus *Hordeum*, and does not share the high β-glucan trait with cultivated barley. In fact, our unpublished studies showed that *H. chilense* and three lines of tritordeum had similar proportions of β-glucan, about 20% of the total AXOS and GOS released by the enzyme fingerprinting method reported here, compared with 60% and 67% for two barley cultivars (unpublished results of A. Lovegrove). Radha et al (2012) also compared AX and β-glucan in tritordeum, wheat, rye, barley and triticale. Quantification of AX using the Uppsala method and of β-glucan using an enzymatic assay gave mean values for 5 genotypes of tritordeum of 6.9 mg/g dry wt. AX and 0.6 mg/g dry wt. of β-glucan, compared with 6.2 and 0.6 mg/g dry wt., respectively, in a single sample of wheat. The higher proportions of AX determined in this study compared with our analyses probably resulted from the presence of complex glucurono-arabinoxylans in the pericarp which are not readily digested by the endoxylanase used for the fingerprinting method. However, tritordeum did differ from bread and durum wheats in having high contents of substituted AXOS which could result in effects on the fermentation in the colon.

Tritordeum is promoted as a novel cereal with health benefits. These claims are partly based on a reported reduction in gluten immunogenic peptides compared to bread wheat, although it is clearly not suitable for those with coeliac disease (Vaquero et al., 2017). However, they are also based on the higher contents of specific types of fibre, minerals and "beneficial phytochemicals" reported here and discussed above.

Two points need to be noted in relation to these suggestions. Firstly, there is substantial variation in the contents of minerals and other bioactive components in grain samples, due to the effects of genotype and environment, and the ranges of contents in tritordeum significantly overlap with those in bread and durum wheats.

Secondly, there is no direct evidence that the differences in contents reported will have long term impacts on the health of consumers, which will depend on the amounts and forms of the grain-based foods consumed (which will determine the bioavailability of bioactive components) and their consumption as part of mixed diets. Long-term intervention and demographic studies are therefore required to provide a valid basis for advice to consumers.

CRediT authorship contribution statement

Peter R. Shewry: Conceptualization, Supervision, Writing – original draft, Project administration, Funding acquisition. **Fred Brouns:** Conceptualization, Supervision, Writing – original draft, Project administration, Funding acquisition. **Jack Dunn:** Investigation. **Jessica Hood:** Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Amanda J. Burridge:** Investigation, Writing – review & editing. **Antoine H.P. America:** Investigation, Writing – review & editing. **Luud Gilissen:** Conceptualization, Writing – review & editing. **Zsuzsan A.M. Proos-Huijsmans:** Investigation, Supervision, Project administration. **Jan Philip van Straaten:** Resources, Writing – review & editing, Funding acquisition. **Daisy Jonkers:** Supervision, Project administration. **Paul A. Lazzeri:** Investigation, Project administration, Writing – review & editing. **Jane L. Ward:** Investigation, Data curation, Supervision, Writing – review & editing. **Alison Lovegrove:** Investigation, Formal analysis, Supervision, Writing – review & editing.

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136312.

References

Alvandi, E., Wong, W. K. M., Joglekar, M. V., Spring, K. J., & Hardikar, A. A. (2022). Short-chain fatty acid concentrations in the incidence and risk-stratification of colorectal cancer: A systematic review and meta-analysis. *BMC Medicine, 20*, 323. https://doi.org/10.1186/s12916-022-02529-4

Andersson, A. A. M., Andersson, R., Piironen, V., Lampi, A.-M., Nystrom, L., … Åman, P. (2013). Contents of dietary fibre components and their relation to associated bioactive components in whole grain wheat samples from the HEALTHGRAIN diversity screen. *Food Chemistry, 136*, 1243–1248. https://doi.org/10.1016/j.foodchem.2012.09.074

Atienza, S. G., Ballesteros, J., Martin, A., & Hornedo-Mendez, D. (2007). Genetic variability of carotenoid concentration and degree of esterification among tritordeum (*xTritordeum* Ascherson et Graebner) and durum wheat accessions. *Journal of Agricultural and Food Chemistry, 55*, 4244–4251. https://doi.org/10.1021/jf070342p

Barrett, J. S., & Gibson, P. R. (2012). Fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) and nonallergic food intolerance: FODMAPs or food chemicals. *Therapeutic Advances in Gastroenterology, 5*, 261–268. https://doi.org/10.1177/1756283X11436241

Călinoiu, L. F., & Vodnar, D. C. (2018). Whole grains and phenolic acids: A review on bioactivity, functionality, health benefits and bioavailability. *Nutrients, 10*, 1615. https://doi.org/10.3390/nu10111615

Corol, D. I., Ravel, C., Raksegi, M., Bedo, Z., Charmet, G., Beale, M. H., Shewry, P. R., & Ward, J. L. (2012). Effects of genotype and environment on the contents of betaine, choline and trigonelline in cereal grains. *Journal of Agricultural and Food Chemistry, 60*, 5471–5481. https://doi.org/10.1021/jf3008794

El Khoury, D., Cuda, C., Luhovyy, B. L., & Anderson, G. H. (2012). Beta glucan: Health benefits in obesity and metabolic syndrome. *Journal of Nutrition and Metabolism, 2012*, Article 851362. https://doi.org/10.1155/2012/851362

Fincher, G. B. (1975). Morphology and chemical composition of barley endosperm cell walls. *Journal of the Institute of Brewing, 81*, 116–122. https://doi.org/10.1002/j.2050-0416.1975.tb03672.x

Gao, L., Wang, S., Oomah, B. D., & Mazza, G. 2002. Wheat quality: Antioxidant activity of wheat millstreams. In P. Ng and C. W. Wrigley (Eds.) *Wheat Quality Elucidation*. (pp. 219–233). AACC International: St. Paul. MN.

Giordano, D., Reyneri, A., Locatelli, M., Coïsson, J. D., & Blandino, M. (2019). Distribution of bioactive compounds in pearled fractions of tritordeum. *Food Chemistry, 301*, Article 125228. https://doi.org/10.1016/j.foodchem.2019.125228

Grausgruber, H., Lovegrove, A., Shewry, R.R. & Bekes, F. (2020) FODMAPs in Wheat. In G. Igrejas, T. Ikeda and C. Guzmán (Eds.) *Wheat quality for improving processing and human health*. (pp517-534) Springer Nature Switzerland AG 2020. (pp. 517-534). 10.1007/978-3-030-34163-3_21.

Haro, C., Guzmán-López, M.H., Marín-Sanz, M., Sánchez-León, S., Vaquero, L., Pastor, J., ,…& Barro. F. (2022) Consumption of tritordeum bread reduces immunogenic gluten intake without altering the gut microbiota. *Foods, 11*, 1439. 10.3390/foods11101439.

Jayachandran M, Chen J, Chung SSM & Xu B. (2018) A critical review on the impacts of β-glucans on gut microbiota and human health. *Journal of Nutritional Biochemistry,61*, 101-110. 0.1016/j.jnutbio.2018.06.010.

Kakabouki, I., Beslemes, D. F., Tigka, E. L., Folina, A., Karydogianni, S., Zisi, C., & Papastylianou, P. (2020). Performance of six genotypes of tritordeum compare to bread wheat under east mediterranean condition. *Sustain, 12*, 9700. https://doi.org/10.3390/su12229700

Li, L., Shewry, P. R., & Ward, J. L. (2008). Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. *Journal of Agriculture and Food Chemistry, 56*, 9732–9739. https://doi.org/10.1021/jf801069s

Lovegrove, A., Wilkinson, M. D., Freeman, J., Pellny, T., Tosi, P., Saulnier, L., … Mitchell, R. A. C. (2013). RNA interference suppression of genes in glycosyl transferase families 43 and 47 in wheat starchy endosperm causes large decreases in arabinoxylan content. *Plant Physiology, 163*, 95–107. https://doi.org/10.1104/pp.113.222653

Mares, D. J., & Stone, B. A. (1973). Studies on wheat endosperm. I. Chemical composition and ultrastructure of the cell walls. *Australian Journal of Biological Science, 26*, 793–812.

Martin, A., Alvarez, J. B., Martin, L. M., Barro, F., & Ballesteros, J. (1999). The development of tritordeum–a novel cereal for food- processing. *Journal of Cereal Science, 30*, 85–95. https://doi.org/10.1006/jcrs.1998.0235

Mottram, D. S., Wedzicha, B. L., & Dodson, A. T. (2002). Acrylamide is formed in the Maillard reaction. *Nature, 419*, 448–449. https://doi.org/10.1038/419448a

Muttucumaru, N., Elmore, J. S., Curtis, T., Mottram, D. S., Parry, M. A. J., & Halford, N. G. (2008). Reducing acrylamide precursors in raw materials derived from wheat and potato. *Journal of Agricultural and Food Chemistry, 56*, 6167–6172. https://doi.org/10.1021/jf800279d

Nitride, C., D'Auria, G., Dente, A., Landolfi, V., Picariello, G., Mamone, G., … Ferranti, P. (2022). Tritordeum as an innovative alternative to wheat: A comparative digestion study on bread. *Molecules, 27*, 1308. https://doi.org/10.3390/molecules27041308

Petrazzani, C., Vanara, F., Bhandari, D. R., Bruni, R., Spengler, B., Blandino, M., & Righetti, L. (2021). 5-*n*-Alkylresorcinol profiles in different cultivars of einkorn, emmer, spelt, common wheat, and tritordeum. *Journal of Agricultural and Food Chemistry, 69*, 14092–14101. https://doi.org/10.1021/acs.jafc.1c05451

Radha, A., Saulnier, L., Aman, P., & Andersson, R. (2012). Enzymic fingerprinting of arabinoxylan and β-glucan in triticale, barley and tritordeum grains. *Carbohydrate Polymers, 90*, 1226-1334. 1.1016/j.carbpol.2012.06.054.

Russo, F., Riezzo, G., Orlando, A., Linslata, M., D'Attoma, B., Prospero, L., & Giannelli, G. (2022). A Comparison of the low-FODMAPs diet and a tritordeum-based diet on the gastrointestinal symptom profile of patients suffering from irritable bowel syndrome-diarrhea variant (IBS-D): A randomized controlled trial. *Nutrients, 14*, 1544. https://doi.org/10.3390/nu14081544

Sánchez-León, S., Haro, C., Villatoro, M., Vaquero, L., Comino, I., … Barro, F. (2020). Tritordeum breads are well tolerated with preference over gluten-free breads in non-celiac wheat-sensitive patients and its consumption induce changes in gut bacteria. *Journal of the Science of Food and Agriculture*. https://doi.org/10.1002/jsfa.10982

Shewry, P. R., Corol, D., Jones, H. D., Beale, M. H., & Ward, J. L. (2017). Defining genetic and chemical diversity in wheat grain by ^{1}H-NMR spectroscopy of polar metabolites. *Molecular Nutrition and Food Research, 61*, 1600807. https://doi.org/10.1002/mnfr.201600807

Suchowilska, E., Kandler, W., Wiwart, M., & Krska, R. (2023). Is Tritordeum (×*Tritordeum martinii* A. Pujadas, nothosp. nov.) grain a potentially useful source of essential minerals in the human diet? *Journal of Food Composition and Analysis, 115*, Article 104874. https://doi.org/10.1016/j.jfca.2022.104874

Suchowilska, E., Wiwart, M., & Przybylska-Balcerek, A. (2021). The profile of bioactive compounds in the grain of various *x Tritordeum* genotypes. *Journal of Cereal Science, 102*, Article 103352. https://doi.org/10.1016/j.jcs.2021.103352

Tian, W., Zheng, Y., Wang, W., Wang, D., Tilley, M., Zhang, G., … Li, Y. (2022). A comprehensive review of wheat phytochemicals: From farm to fork and beyond. *Comprehensive Reviews of Food Science and Food Safety, 21*, 2274–2308. https://doi.org/10.1111/1541-4337.12960

Tiihonen, K., Riihinen, K., Lyyra, M., Sarkkinen, E., Craig, S. A. S., & Tenning, P. (2014). Authorised EU health claims for betaine. In M. J. Sadler (Ed.), *Foods, nutrients and food ingredients with authorised EU health claims* (pp. 251–273). Woodhead Publishing. https://doi.org/10.1533/9780857098481.4.251.

Turner, A. L., Michaelson, L. V., Shewry, P. R., Lovegrove, A., & Spencer, J. P. E. (2021). Increased bioavailability of phenolic acids and enhanced vascular function following intake of feruloyl esterase-processed high fibre bread: A randomized, controlled, single blind, crossover human intervention trial. *Clinical Nutrition, 40*, 788–793. https://doi.org/10.1016/j.clnu.2020.07.026

Vaquero, L., Comino, I., Vivas, S., Rodríguez-Martín, L., Giménez, M. J., Pastor, J., … Barro, F. (2017). Tritordeum: A novel cereal for food processing with good acceptability and significant reduction in gluten immunogenic peptides in comparison with wheat. *Journal of the Science of Food and Agriculture, 98*, 2201–2209. https://doi.org/10.1002/jsfa.8705

Venegas, D. P., De la Fuente, M. K., Landskron, G., Gonzalez, M. J., Quera, R., Dijkstra, G., … Hermoso, M. A. (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel disease. *Frontiers in Immunology, 10*, 277. https://doi.org/10.3389/fimmu.2019.00277

Visioli, G., Lauro, M., Vamerali, T., Dal Cortivo, C., Panozzo, A., Folloni, S., … Ranieri, R. (2020). A comparative study of organic and conventional management on the rhizosphere microbiome, growth and grain quality traits of tritordeum. *Agronomy, 10*, 1717. https://doi.org/10.3390/agronomy10111717

Ward, J., Poutanen, K., Gebruers, K., Piironen, V., Lampi, A.-M., Nyström, L., … Shewry, P. R. (2008). The HEALTHGRAIN cereal diversity screen: Concept, results and prospects. *Journal of Agricultural and Food Chemistry, 56*, 9699–9709. https://doi.org/10.1021/jf8009574

Yding E. D., Pagenstecher, M., Trummer, J., Aleksander Poreda, A., Mogens L. & Andersen, M. L., Jespersen, B. M. (2022). Effect of malting regimes on the malt quality of tritordeum for beer brewing. *European Food Research and Technology*. 10.1007/s00217-022-04136-2.

Young, I. S., & Woodside, J. V. (2001). Antioxidants in health and disease. *Journal of Clinical Pathology, 54*, 176–186. https://doi.org/10.1136/jcp.54.3.176

Yousfi S., Serret M. D., Voltas J. & Araus, J. L. (2010). Effect of salinity and water stress during the reproductive stage on growth, ion concentrations, Δ^{13}C, and δ^{15}N of durum wheat and related amphiploids. *Journal of Experimental Botany*, 61, 3529–3542.

Zdaniewicz, M., Pater, A., Hrabia, O., Dulíński, R., & Cioch-Skoneczny, M. (2020). Tritordeum malt: An innovative raw material for beer production. *Journal of Cereal Science, 96*, Article 103095. https://doi.org/10.1016/j.jcs.2020.103095

Zheng, X., Levine, D., Shen, J., Gogarten, S. M., Laurie, C., & Weir, B. S. (2012). A high performance computing toolset for relatedness and principal component analysis of SNP data. *Bioinformatics, 28*, 3326–3328. https://doi.org/10.1093/bioinformatics/bts606