

Article

Yeast-Mediated Pre-Fermentation Modulates the Fecal Microbiome In Vitro in a Genotype-Dependent Manner

Tekmile Cankurtaran Kömürcü ^{1,2}, Miguel Angel Alvarez Gonzales ² , İhsan Sarioğlu ¹,
Stephen R. Lindemann ^{2,3,4}  and Yunus Emre Tuncil ^{1,5,*} 

¹ Department of Food Engineering, Faculty of Engineering, Necmettin Erbakan University, 42040 Konya, Türkiye; tekmilecankurtaran@gmail.com (T.C.K.); ihsansarioglan@hotmail.com (İ.S.)

² Whistler Center for Carbohydrate Research, Department of Food Science, Purdue University, West Lafayette, IN 47907, USA; gonzale1@purdue.edu (M.A.A.G.); lindems@purdue.edu (S.R.L.)

³ Department of Nutrition Science, Purdue University, West Lafayette, IN 47907, USA

⁴ Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA

⁵ Medical and Cosmetic Plants Application and Research Center, Necmettin Erbakan University, 42090 Konya, Türkiye

* Correspondence: ytuncil@erbakan.edu.tr

Abstract

This study investigated the effects of yeast-mediated pre-fermentation on the composition and functional characteristics of modern and ancient wheat genotypes, and their subsequent impact on gut microbial fermentation. Whole wheat flours were pre-fermented using *Saccharomyces cerevisiae* (6%, w/w) at 30 °C for 6 h. Raw and pre-fermented whole wheat samples were subjected to in vitro upper gastrointestinal digestion, followed by monosaccharide composition analysis, in vitro fecal fermentation using a pooled inoculum prepared from three healthy donors, microbial profiling, and short-chain fatty acid (SCFA) quantification. Fermentation increased Folin–Ciocalteu-reactive values and antioxidant capacity, while reducing residual starch and protein after digestion, indicating alterations in the composition of digestion-derived residues. After fermentation by *S. cerevisiae*, monosaccharide analysis revealed a relative enrichment of glucose, accompanied by decreased proportions of arabinose and xylose. Following in vitro fecal fermentation, pre-fermented wheat samples exhibited significantly ($p < 0.001$, AMOVA) different microbial community structures compared with their raw counterparts, based on Bray–Curtis dissimilarities, whereas differences in α -diversity were comparatively limited. In addition, taxa associated with carbohydrate fermentation and SCFA production, including *Parabacteroides distasonis*, *Butyrivibrio crossotus*, and *Kineothrix*, showed treatment- and genotype-dependent responses. All wheat samples supported SCFA production, although SCFA accumulation was more gradual than with inulin. Overall, yeast-mediated pre-fermentation was associated with changes in wheat composition and subsequent shifts in fecal microbial community structure and fermentation dynamics, with responses varying among wheat genotypes.

Keywords: ancient and modern wheat; in vitro fecal fermentation; 16S rRNA sequencing; short-chain fatty acids; phenolic compounds; antioxidant capacity



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1. Introduction

Whole grains are increasingly recognized as important dietary components due to their rich content of dietary fiber, phenolic compounds, and other bioactive constituents

that contribute to human health. Regular consumption of whole grain cereals has been associated with reduced risk of chronic diseases, including cardiovascular disease, type 2 diabetes, and colorectal cancer [1]. These health-promoting effects are largely attributed to the presence of non-digestible carbohydrates, particularly arabinoxylans and β -glucans, which play a central role in modulating gut microbiota composition and metabolic activity [2–4].

Wheat dietary fibers are structurally complex and heterogeneous, consisting mainly of arabinoxylans characterized by varying degrees of substitution and branching. The physicochemical properties of these fibers, including solubility, molecular weight, and branching pattern, strongly influence their fermentability and accessibility to gut microbiota [3,4]. This structural complexity is considered as an important factor influencing the extent to which dietary fibers can be accessed and utilized by gut microbiota. However, a substantial fraction of these carbohydrates remains embedded within the plant cell wall matrix or associated with proteins and starch, limiting their bioaccessibility and subsequent fermentation in the colon [5].

Food processing techniques have been shown to significantly alter the organization and physicochemical properties of dietary fibers and thereby influence their functional properties. Among these, fermentation is gaining increasing attention as a promising strategy to enhance the nutritional and functional quality of cereal-based foods [6,7]. Microbial and enzymatic activities during fermentation can lead to partial depolymerization of cell wall polysaccharides, release of bound phenolic compounds, and modification of starch–protein interactions [8–10]. In particular, yeast-mediated fermentation has been reported to improve the accessibility of macronutrients and modify the physicochemical properties of cereal matrices, potentially enhancing digestibility and microbial fermentability [6]. However, the impact of yeast-mediated pre-fermentation, particularly when applied as a pre-treatment prior to digestion, on the composition of the resulting digestion-derived residues and their subsequent microbial utilization remains insufficiently understood. Previous studies have focused primarily on compositional changes induced by fermentation [9,10], with comparatively less attention to how these changes relate to subsequent gut microbial responses. Furthermore, differences between ancient and modern wheat genotypes in terms of fiber composition, cell wall architecture, and resistance to processing may lead to distinct fermentation outcomes, yet comparative studies addressing these aspects are still limited [11,12].

In this context, the present study aimed to investigate compositional changes in digestion-derived wheat residues following yeast-mediated pre-fermentation of both modern and ancient wheat genotypes and to evaluate their associations with subsequent gut microbial composition and metabolic activity. To achieve this, whole wheat flours were pre-fermented using baker's yeast (*Saccharomyces cerevisiae*) (6%, *w/w*) at 30 °C for 6 h. *S. cerevisiae* is widely used in cereal fermentations, and microbial fermentation, including fermentation by *S. cerevisiae*, has been reported to induce compositional and physicochemical changes in the cereal matrix through endogenous grain enzyme activity and microbial metabolism [9,13]. It is noteworthy to mention here that the term “modern wheat” refers to the commercially cultivated species *T. aestivum*, *T. durum*, and *T. compactum*, whereas “ancient wheat” refers to the historically cultivated species *T. monococcum*, *T. dicoccum*, and *T. spelta*. These terms are used solely as operational groupings for comparative purposes and do not imply that either category represents a genetically homogeneous group.

Rather than producing a final food product (i.e., reproducing a bread-making process), the present study employed a simplified laboratory pre-fermentation model to evaluate the effects associated with the yeast-mediated pre-fermentation process on wheat composition before simulated gastrointestinal digestion and *in vitro* fecal fermentation. This approach minimizes the influence of technological factors such as dough development,

gluten network formation, proofing, and baking, thereby enabling a controlled evaluation of compositional changes associated with the pre-fermentation process and their relationship with subsequent fermentability. Raw and pre-fermented wheat samples were subjected to simulated upper gastrointestinal digestion to obtain digestion-derived residues, followed by monosaccharide composition analysis, in vitro fecal fermentation, microbial community profiling, and SCFA quantification. By integrating compositional, microbiological, and metabolic analyses, this study explores potential associations between the composition of digestion-derived wheat residues, gut microbial responses, and SCFA production.

2. Materials and Methods

2.1. Materials

Triticum aestivum L. (cv. Esperia), *Triticum durum* Desf. (cv. Kızıltan-91), *Triticum compactum* Host (cv. Karaduman) and *Triticum spelta* L. (spelt) were obtained from Konya, Türkiye; *Triticum monococcum* L. (einkorn) was obtained from Kastamonu, Türkiye; and *Triticum dicoccum* Schrank ex Schübl. (emmer) was obtained from Kars, Türkiye. All wheat samples were harvested in 2023. For the production of whole wheat flour, samples were milled ($<500\ \mu\text{m}$) using a hammer mill (Laboratory Mill 3100, Perten Instruments AB, Stockholm, Sweden) with a 100% extraction rate.

2.2. Pre-Fermentation of Wheat Samples by Yeast

The flour samples were mixed with distilled water at a ratio of 1:15 (w/v) to ensure complete hydration and homogeneous dispersion of the flour particles and yeast cells throughout the fermentation medium. This slurry system was intentionally selected as a simplified laboratory model to provide uniform fermentation conditions and to minimize mass-transfer limitations associated with dough structure. Baker's yeast (*Saccharomyces cerevisiae*) was added at 6% (w/w), corresponding to approximately 6×10^7 CFU/g of flour. Fermentation was carried out in closed containers at 30 °C for 6 h in a temperature-controlled shaking water bath (ST30; Nüve, Ankara, Türkiye). At the end of fermentation, the resulting slurry was dried in a convection oven (KD200; Nüve, Ankara, Türkiye) at 50 °C until the moisture content decreased to approximately 10%. The dried samples were subsequently re-milled ($<500\ \mu\text{m}$) to obtain fermented whole wheat flour. Raw wheat flour samples were not subjected to hydration, incubation, drying, or re-milling and were analyzed in their original untreated form.

2.3. Chemical Composition

Proximate composition (moisture, ash, protein, and fat) was determined according to standard AACC methods [14]. Moisture content was measured by oven drying at 135 °C to constant weight, ash content by incineration at 550 °C in a muffle furnace, protein content by the Kjeldahl method (Kjeltec™ 8200, FOSS, Hillerød, Denmark) and fat content using a solvent extraction system (SER 148/6, VELP Scientifica Srl, Velp Scientifica, Usmate, Italy).

2.4. Determination of Total Phenolic Content (TPC) and Antioxidant Capacity

TPC was determined using the Folin–Ciocalteu assay following Singleton and Rossi [15], with slight modifications [16]. The Folin–Ciocalteu assay was used to estimate Folin–Ciocalteu-reactive compounds, expressed as apparent total phenolic content. Because the assay is based on reducing capacity and is not specific to phenolic compounds, non-phenolic reducing substances present in the extracts may also contribute to the measured response [17]. Ground samples (1 g) were treated with 10 mL of methanol–water solution (80:20, v/v) acidified with 1% HCl. Extraction was performed in a shaking water bath (ST-402; Nüve, Ankara, Türkiye) at 24 ± 1 °C for 2.5 h. The extracts were then cen-

trifuged (Z 326 K, HERMLE Labortechnik GmbH, Wehingen, Germany) (3000 rpm, 10 min), and the resulting supernatant was used for analysis. For the assay, 0.1 mL of extract was mixed with 0.5 mL of Folin–Ciocalteu reagent (10%, *v/v*) and 0.4 mL of sodium carbonate solution (7.5%, *w/v*). The mixture was incubated in the dark at room temperature for 2 h, and absorbance was measured at 760 nm using a spectrophotometer (U-1800, Hitachi High-Technologies Corporation, Tokyo, Japan). Results were expressed as gallic acid equivalents (GAE) per kg of dry sample.

Three different methods (DPPH, FRAP and CUPRAC) were used for antioxidant activity analysis of the samples. The DPPH (2,2-diphenyl-1-picrylhydrazyl) value of the extracts of the samples was analyzed according to the method described by Beta et al. [18]. The FRAP (ferric reducing antioxidant power) analysis of samples was carried out according to the method of Gao et al. [19]. The method of Apak et al. [20] was used in the determination of antioxidant activity with the CUPRAC (cupric ion reducing antioxidant capacity) method. Results of all antioxidant assays were expressed as μmol Trolox equivalents (TE)/g dry matter to facilitate comparison among assays. All measurements were performed in triplicate. All results were expressed on a dry-matter basis. No correction for processing-related mass loss was applied because the analyses were performed on dried samples under identical extraction conditions.

2.5. Simulation of Upper Gastrointestinal Tract Digestion

In vitro upper gastrointestinal digestion was performed following the method of Tunçil et al. [21], with additional methodological details described below. Wheat flour samples were suspended in distilled water (1:12, *w/v*), gelatinized by boiling for 10–20 min, cooled to 37 °C, and adjusted to pH 2.5. Gastric digestion was performed using pepsin (Sigma P7000, $\geq 250 \text{ U mg}^{-1}$; prepared at 100 mg mL^{-1}) for 30 min at 37 °C under continuous stirring (700 rpm). The pH was then adjusted to 6.9 ± 0.1 , and intestinal digestion was carried out using pancreatin (Sigma P7545; $8\times \text{ USP}$, prepared at 125 mg mL^{-1}) and amyloglucosidase (Megazyme E-AMGDF, 3260 U mL^{-1} ; 0.5 mL) for 6 h at 37 °C under continuous stirring. No bile salts were included in this protocol. Enzymes were inactivated by boiling for 20 min. The pH was adjusted to 7.0, and three volumes of ethanol were added to recover the non-digestible residue, which was precipitated overnight at 4 °C, filtered, washed twice with 80% ethanol, dried in a vacuum oven (40 °C), ground, and stored at -20 °C until further analysis. The purpose of the simulated upper gastrointestinal digestion was to reduce digestible starch and protein fractions and recover digestion-derived residues for subsequent in vitro fecal fermentation.

2.6. Determination of Monosaccharide Composition

Neutral monosaccharide composition of the digestion-derived residues was determined as their alditol acetate derivatives using GC/MS (GC–MS, Agilent A6890; Agilent Technologies, Santa Clara, CA, USA), based on established protocols [22] with modifications described previously [21]. Briefly, samples (15 mg) were hydrolyzed with 72% sulfuric acid at room temperature for 1 h with intermittent mixing, followed by dilution with distilled water and further hydrolysis at 100 °C for 3 h. After cooling, the hydrolysates were neutralized with ammonia. An aliquot of the hydrolysate was transferred to a clean tube, spiked with inositol as an internal standard, and dried under a stream of nitrogen. The dried residues were reduced using sodium borodeuteride (NaBD_4) in ammonium hydroxide solution, followed by acetylation with acetic anhydride to form alditol acetate derivatives. The derivatives were extracted using dichloromethane (DCM), washed with water, and the organic phase was dried. The final residue was dissolved in acetone and

analyzed by GC/MS for determination of monosaccharide composition. GC/MS operating conditions were applied according to Tuncil et al. [21].

2.7. *In Vitro Fecal Fermentation*

In vitro fecal fermentation was performed to evaluate the fermentability of digestion-derived residues obtained from raw and fermented wheat samples following upper gastrointestinal digestion, based on a previously described protocol [23] with minor modifications. All procedures were conducted under anaerobic conditions using an anaerobic chamber (BACTRON300; Shel Lab, Cornelius, NC, USA) with a gas composition of 90% N₂, 5% H₂, and 5% CO₂. Carbonate–phosphate buffer was prepared, sterilized by autoclaving (121 °C, 20 min), supplemented with cysteine hydrochloride (0.25 g/L) as a reducing agent, and equilibrated overnight in the anaerobic chamber to ensure oxygen removal. Digested samples (25 mg) were transferred into 25 mL Balch tubes in triplicate for each treatment and fermentation time point (6, 12, 24, and 48 h). The triplicate tubes represented independent technical/process fermentation replicates prepared from the same pooled fecal inoculum. Inulin was included as a rapidly fermentable positive control, while tubes without substrate served as negative controls. Each tube received 2 mL of pre-reduced buffer. Fresh fecal samples were obtained from three healthy donors (one female and two males; aged 27–37 years) with no history of prebiotic or probiotic use during the preceding six months and with normal body mass index. Immediately after collection, the fecal samples were homogenized with buffer (1:3, *w/v*) and filtered through four layers of cheesecloth. Then equal volumes of fecal slurry from each donor were pooled to prepare a pooled inoculum. Aliquots (0.5 mL) of the pooled fecal slurry were used to inoculate each fermentation tube. The same pooled inoculum was used for all treatments and fermentation time points.

Fermentation was carried out at 37 °C, and samples were collected at 0, 6, 12, 24, and 48 h. At each time point, aliquots were taken for DNA extraction (1.6 mL) and SCFA analysis (0.8 mL). For SCFA analysis, an internal standard solution (200 µL), prepared according to the protocol of Tuncil et al. [21], was added to each sample. The mixture was vortexed and stored at −20 °C until analysis. Ethical approval for the collection and use of human fecal samples was obtained from the Necmettin Erbakan University Health Sciences Scientific Research Ethics Committee (Approval date: 7 June 2023; decision number: 14580).

2.8. *Short-Chain Fatty Acid (SCFA) Analysis*

SCFAs produced during in vitro fecal fermentation of raw and fermented wheat samples were quantified using GC coupled with a flame ionization detector (FID; Shimadzu GC2030, Kyoto, Japan) equipped with a fused silica capillary column (Stabilwax-DA; Restek Corporation, Bellefonte, PA, USA) as previously described [21]. Frozen samples were thawed and centrifuged at 13,000 rpm for 10 min. The supernatants were directly injected (4 µL) into the GC system. Quantification was performed using external standards of acetate, propionate, and butyrate (Sigma-Aldrich, St. Louis, MO, USA). Results were expressed as mM concentrations of individual SCFAs, and total SCFA was calculated as the sum of these components.

2.9. *DNA Extraction, 16S rRNA Sequencing, and Bioinformatic Analysis*

Samples collected at selected time points (0 and 24 h) were thawed at room temperature and centrifuged (13,000 rpm, 10 min, 4 °C). After removal of the supernatant, genomic DNA was extracted from the pellet using a chemoenzymatic lysis approach based on previously established protocols [24], combined with bead-beating phenol–chloroform extraction optimized for fecal samples [25]. Detailed DNA extraction procedures have been described previously by Yaşar [26]. The V4–V5 region of the 16S rRNA gene was amplified using universal primers (515-FB and 926-R), and sequencing libraries were prepared using

TruSeq barcoding, as previously described by Tuncil et al. [21]. Barcoded amplicons were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) and pooled. The pooled samples were then sequenced on an Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) (2×250 bp, V2 chemistry) at the Purdue University Genomic Core Facility (West Lafayette, IN, USA).

Raw sequence data were processed using Mothur (v1.48.0) according to the MiSeq standard operating procedure (https://mothur.org/wiki/miseq_sop/, accessed on 30 August 2026) [27,28]. During quality filtering, sequences were restricted to a maximum length of 411 nt, with no ambiguous bases allowed and a maximum homopolymer length of 8 nt. The filtered sequences were subsequently aligned against the mothur-formatted SILVA reference alignment [29]. Taxonomic classification was carried out using version 18 of the mothur-formatted Ribosomal Database Project (RDP) training set [30], with classification performed to the species level using a minimum bootstrap threshold of 97%. Sequences that could not be classified, as well as non-bacterial sequences originating from eukaryotes, mitochondria, or chloroplasts, were excluded from subsequent analyses. To standardize sequencing depth across samples prior to the calculation of ecological diversity metrics, each sample was subsampled to 6961 reads. Microbial community α -diversity was assessed using the nseqs, coverage, invsimpson, simpson even, chao, and shannon calculators through the summary.single() command in mothur. β -diversity was evaluated using the Bray–Curtis distance metric implemented through the dist.shared() command in mothur [31].

2.10. Statistical Analysis

Statistical analyses and data visualizations were performed using GraphPad Prism (version 10; GraphPad Software, Boston, MA, USA). Results are expressed as mean $\pm$ standard deviation (SD), unless otherwise stated. For endpoints evaluated according to wheat genotype and pre-fermentation status, two-way ANOVA was performed using genotype (six levels) and pre-fermentation status (untreated vs. fermented) as fixed factors, including their interaction. Tukey's multiple comparison test was used for post hoc comparisons at $\alpha = 0.05$. Main-effect and interaction F and p values are reported in Supplementary Table S1. The modern-versus-ancient classification was used descriptively and was not treated as an independent inferential factor. For SCFA outcomes, two-way ANOVA was performed at the 24-h time point using genotype and pre-fermentation status as fixed factors, including their interaction. For β -diversity analysis, statistical significance was assessed using analysis of molecular variance (AMOVA) implemented in Mothur.

3. Results and Discussion

3.1. Proximate Composition of Wheat Samples

The proximate composition of wheat samples, including moisture, ash, protein, and fat contents, is presented in Figure 1. Two-way ANOVA revealed significant main effects of both wheat genotype and pre-fermentation on all proximate composition parameters (Supplementary Table S1). Significant genotype $\times$ pre-fermentation interactions were observed for moisture ($p = 0.0003$), protein ($p = 0.0188$), and fat ($p = 0.0266$), whereas the interaction was not significant for ash ($p = 0.5392$). Thus, the magnitude of the response to pre-fermentation differed among wheat genotypes for moisture, protein, and fat, whereas the effect on ash was comparatively consistent across genotypes.

Fermented samples generally exhibited lower moisture contents than their raw counterparts, whereas differences in ash, protein, and fat contents were comparatively smaller. Ancient wheat species tended to exhibit higher ash and protein contents than modern wheat, consistent with previously reported compositional differences among wheat species [32,33].

Because the observed differences may reflect changes in the relative composition of the processed samples, they should not be interpreted as direct evidence of synthesis or degradation of individual components during pre-fermentation.

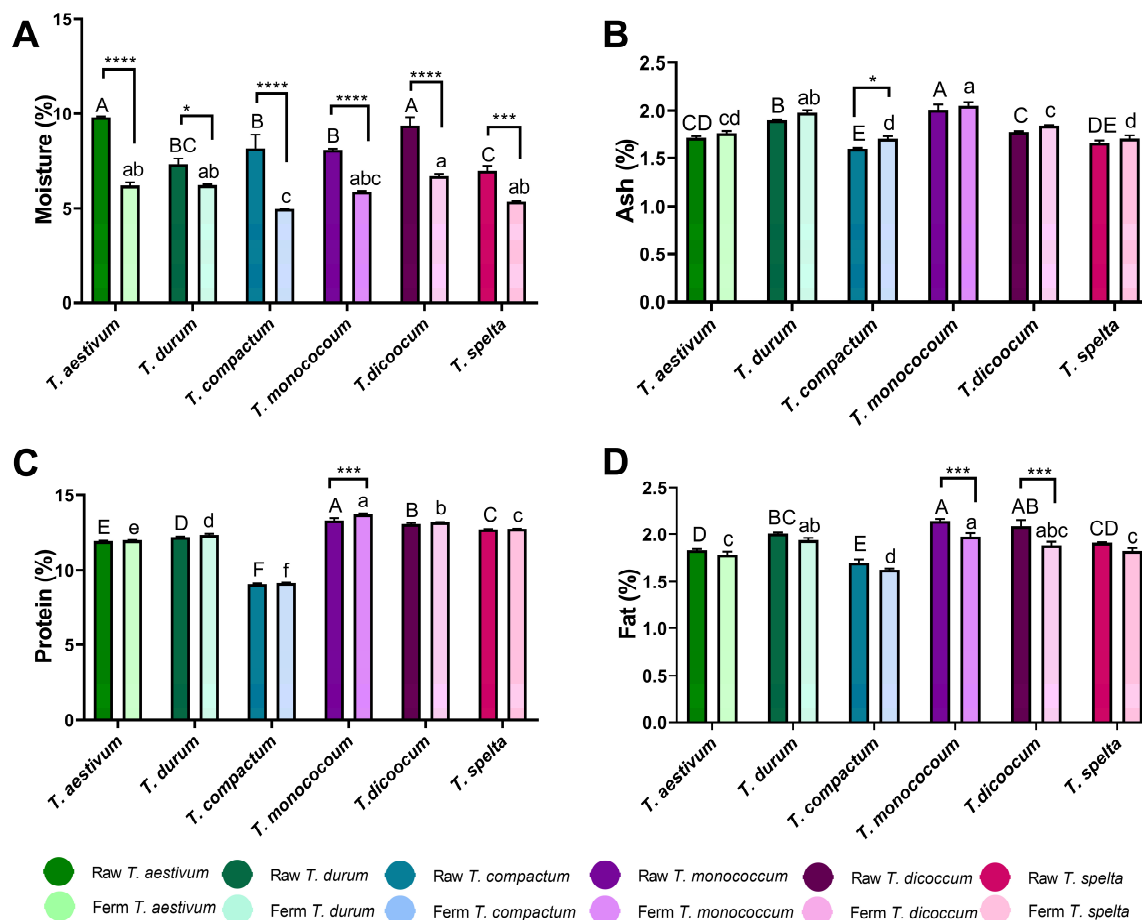


Figure 1. Proximate composition of raw and fermented wheat samples. (A) moisture; (B) ash; (C) protein; and (D) fat contents. Values are expressed as mean $\pm$ SD ($n = 3$). Uppercase letters indicate statistically significant differences among raw samples across wheat genotypes, whereas lowercase letters indicate statistically significant differences among fermented samples across wheat genotypes. Asterisks indicate significant differences between raw and fermented samples within each wheat genotype (* $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$). Multiple comparisons were performed using Tukey's multiple comparison test following two-way ANOVA. Results of the two-way ANOVA evaluating the main effects of genotype, pre-fermentation, and their interaction are provided in Supplementary Table S1.

3.2. Total Phenolic Content (TPC) and Antioxidant Capacity

The TPC and antioxidant capacities of wheat samples are presented in Figure 2. Statistical analysis demonstrated significant main effects of both wheat genotype and pre-fermentation on TPC and all antioxidant capacity assays ($p < 0.0001$; Supplementary Table S1). Significant genotype $\times$ pre-fermentation interactions were also observed for TPC ($p < 0.0001$), DPPH ($p = 0.0344$), FRAP ($p < 0.0001$), and CUPRAC ($p < 0.0001$), indicating that the magnitude of the response to pre-fermentation varied among wheat genotypes. Overall, both wheat genotype and pre-fermentation significantly influenced Folin–Ciocalteu-reactive values and antioxidant capacity. TPC showed noticeable variation among wheat genotypes, with ancient wheat varieties (*T. monococcum*, *T. dicoccum*, and *T. spelta*) generally exhibiting higher values compared with modern wheat (*T. aestivum*, *T. durum*, and *T. compactum*). This trend is consistent with previous studies reporting differences in phenolic constituents and antioxidant activity among ancient and modern wheat species [32,34,35]. Pre-fermentation

resulted in higher Folin–Ciocalteu-reactive values in most samples. These differences may reflect changes in the extractability of phenolic compounds and/or other reducing constituents during fermentation [10,36,37]. However, because free and bound phenolic fractions and chromatographic phenolic profiles were not determined, the underlying mechanisms cannot be established from the present data.

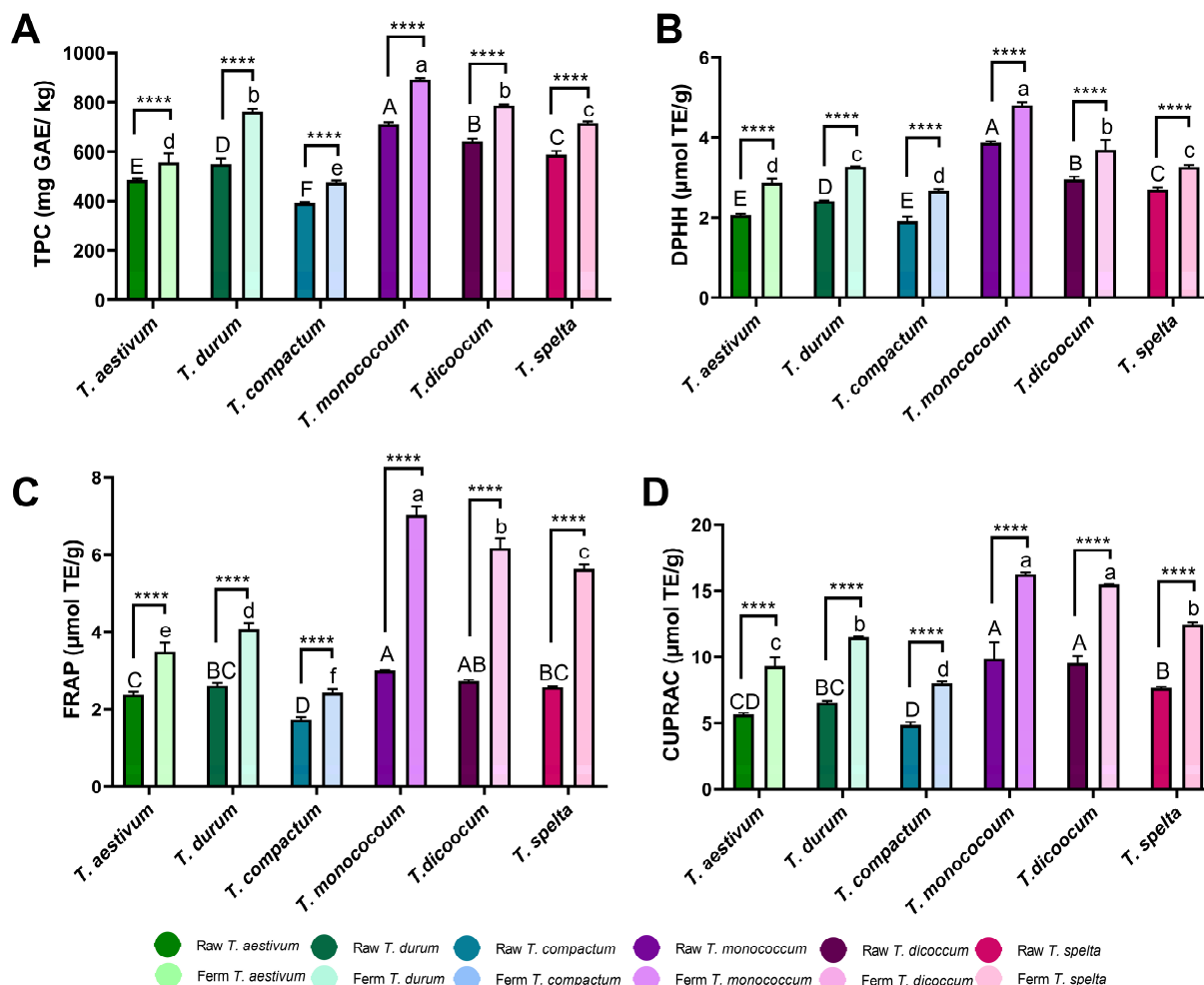


Figure 2. TPC and antioxidant capacity of raw and fermented wheat samples. (A) TPC; (B) DPPH; (C) FRAP; and (D) CUPRAC. Values are expressed as mean $\pm$ SD ($n = 3$). Uppercase letters indicate statistically significant differences among raw samples across wheat genotypes, whereas lowercase letters indicate statistically significant differences among fermented samples across wheat genotypes. Asterisks indicate significant differences between raw and fermented samples within each wheat genotype (**** $p < 0.0001$). Multiple comparisons were performed using Tukey’s multiple comparison test following two-way ANOVA. Results of the two-way ANOVA evaluating the main effects of genotype, pre-fermentation, and their interaction are provided in Supplementary Table S1.

A similar trend was observed for antioxidant activity assays. DPPH, FRAP, and CUPRAC values were generally higher in fermented samples than in their raw counterparts, although the magnitude of these differences varied among wheat genotypes. The ancient wheat species examined in this study generally exhibited higher antioxidant capacities, which may be associated with differences in Folin–Ciocalteu-reactive compounds and other antioxidant constituents among wheat genotypes [32,34,35,38]. However, the specific compounds responsible for these differences were not identified in the present study. The genotype-dependent variation in the response to pre-fermentation is consistent with the significant genotype $\times$ pre-fermentation interactions observed for TPC, DPPH, FRAP, and

CUPRAC (Supplementary Table S1). Thus, although pre-fermentation had a significant overall effect on all four antioxidant-related parameters, the magnitude of this effect depended on wheat genotype.

3.3. Residual Starch and Protein After Upper Gastrointestinal Digestion

To evaluate the composition of the residues obtained after simulated upper gastrointestinal digestion, residual starch and protein contents of the samples were determined (Figure 3). Statistical analysis demonstrated significant main effects of both wheat genotype and pre-fermentation on residual starch and protein contents ($p < 0.0001$; Supplementary Table S1). Significant genotype $\times$ pre-fermentation interactions were also observed for both parameters ($p < 0.0001$), indicating that the effect of pre-fermentation on the composition of the recovered digestion-derived residues differed among wheat genotypes. Residual starch remained detectable across all samples, indicating the presence of starch fractions that were not hydrolyzed under the applied digestion conditions. Such residual starch may reflect limited enzymatic accessibility associated with the structural organization of starch within the cereal matrix, including its entrapment within cell wall networks or interactions with proteins and dietary fiber components, which can limit enzyme accessibility [39,40].

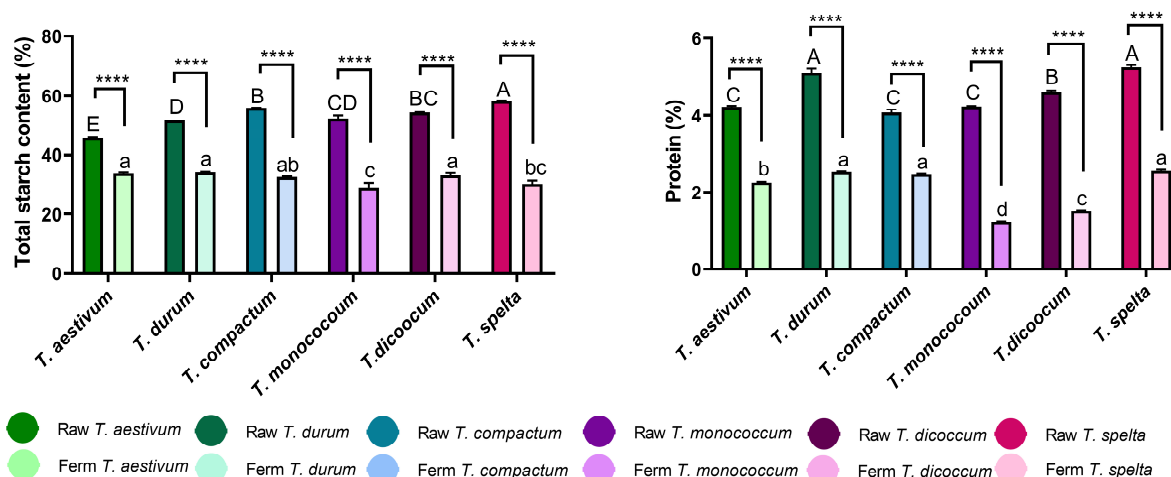


Figure 3. Residual starch and protein contents of raw and fermented wheat samples after simulated upper gastrointestinal digestion. Values are expressed as mean $\pm$ SD ($n = 3$). Uppercase letters indicate statistically significant differences among raw samples across wheat genotypes, whereas lowercase letters indicate statistically significant differences among fermented samples across wheat genotypes. Asterisks indicate significant differences between raw and fermented samples within each wheat genotype (**** $p < 0.0001$). Multiple comparisons were performed using Tukey's multiple comparison test following two-way ANOVA. Results of the two-way ANOVA evaluating the main effects of genotype, pre-fermentation, and their interaction are provided in Supplementary Table S1.

Protein content was lower in the recovered digested wheat residues than in the corresponding undigested wheat samples (Figure 1), consistent with protein hydrolysis during simulated gastrointestinal digestion. Residual protein after digestion may partly reflect limited enzymatic accessibility associated with interactions between dietary fiber and the protein matrix or physical entrapment within the cereal structure [41].

Fermented samples exhibited lower residual starch and protein contents than their corresponding raw samples across all wheat genotypes (Figure 3). Consistent with the significant genotype $\times$ pre-fermentation interactions (Supplementary Table S1), the magnitude of these differences varied among wheat genotypes. These findings indicate that pre-fermentation was associated with genotype-dependent differences in the composition of the residues recovered after digestion. Such differences may reflect fermentation-associated

changes in the cereal matrix that influenced starch and protein hydrolysis during digestion. However, because a matched non-inoculated processed control was not included, the individual contributions of hydration, incubation, endogenous cereal enzyme activity, drying, and yeast fermentation cannot be distinguished. Therefore, these results should be interpreted as the effects associated with the overall pre-fermentation process rather than as changes attributable exclusively to yeast activity. The resulting differences in the composition of the recovered digestion-derived residues may subsequently influence their utilization during colonic fermentation.

3.4. Monosaccharide Composition of Digestion-Derived Residues

To characterize the carbohydrate composition of the recovered digestion-derived residues, wheat samples subjected to *in vitro* digestion were analyzed for their monosaccharide profiles (Figure 4). Glucose, arabinose, and xylose were the predominant monosaccharides across all samples, consistent with the presence of glucose-, arabinose-, and xylose-containing carbohydrate fractions in the recovered digestion-derived residues. Pre-fermentation was associated with changes in the relative monosaccharide composition of these residues. Two-way ANOVA demonstrated significant main effects of wheat genotype on all monosaccharides except galactose (Supplementary Table S1). Pre-fermentation significantly affected arabinose, xylose, mannose, glucose, and the Ara/Xyl ratio, whereas no significant main effects were observed for rhamnose, fucose, or galactose. Significant genotype $\times$ pre-fermentation interactions were detected for fucose, arabinose, xylose, mannose, glucose, and the Ara/Xyl ratio, indicating that the compositional response to pre-fermentation depended on wheat genotype for these components. Arabinose and xylose are characteristic constituents of arabinoxylans, whereas glucose may originate from cellulose, β -glucans, residual starch, starch-derived glucans, or other glucose-containing polysaccharides [42,43], as well as potentially from residual yeast-derived glucans remaining after the fermentation and digestion procedures.

Pre-fermentation was associated with a consistent shift in the relative monosaccharide composition across all wheat genotypes, increasing the relative proportion of glucose from 31.4 to 51.3% in raw samples to 55.6–63.0% after fermentation, while reducing arabinose from 23.4 to 38.4% to 18.1–22.3% and xylose from 18.6 to 24.6% to 11.4–13.9%. This trend was observed in both modern and ancient wheat genotypes, although its magnitude differed among genotypes, in agreement with the significant genotype $\times$ pre-fermentation interactions observed for arabinose, xylose, glucose, and the Ara/Xyl ratio (Supplementary Table S1). Importantly, because the data represents relative monosaccharide composition, the higher glucose proportion should be interpreted as relative enrichment rather than evidence of absolute glucose accumulation. Because the GC/MS analysis quantified monosaccharides after complete acid hydrolysis and no yeast-only control was included, the relative contributions of wheat- and yeast-derived carbohydrates could not be distinguished. Moreover, monosaccharide profiling does not directly demonstrate molecular debranching, changes in glycosidic linkage patterns, molecular-weight distribution, or other structural characteristics of arabinoxylans. Therefore, these results should be interpreted as compositional characteristics of the pre-fermented wheat samples rather than definitive evidence of structural modification of wheat-derived dietary fiber. The observed changes are compatible with several possible processes, including changes in arabinoxylan extractability, preferential loss of arabinose-rich fractions, endogenous cereal enzyme activity during fermentation, potential contributions from residual yeast biomass, or shifts in the relative abundance of different carbohydrate fractions. Related processing-associated changes in cereal arabinoxylan composition and properties have been reported previously [44–47].

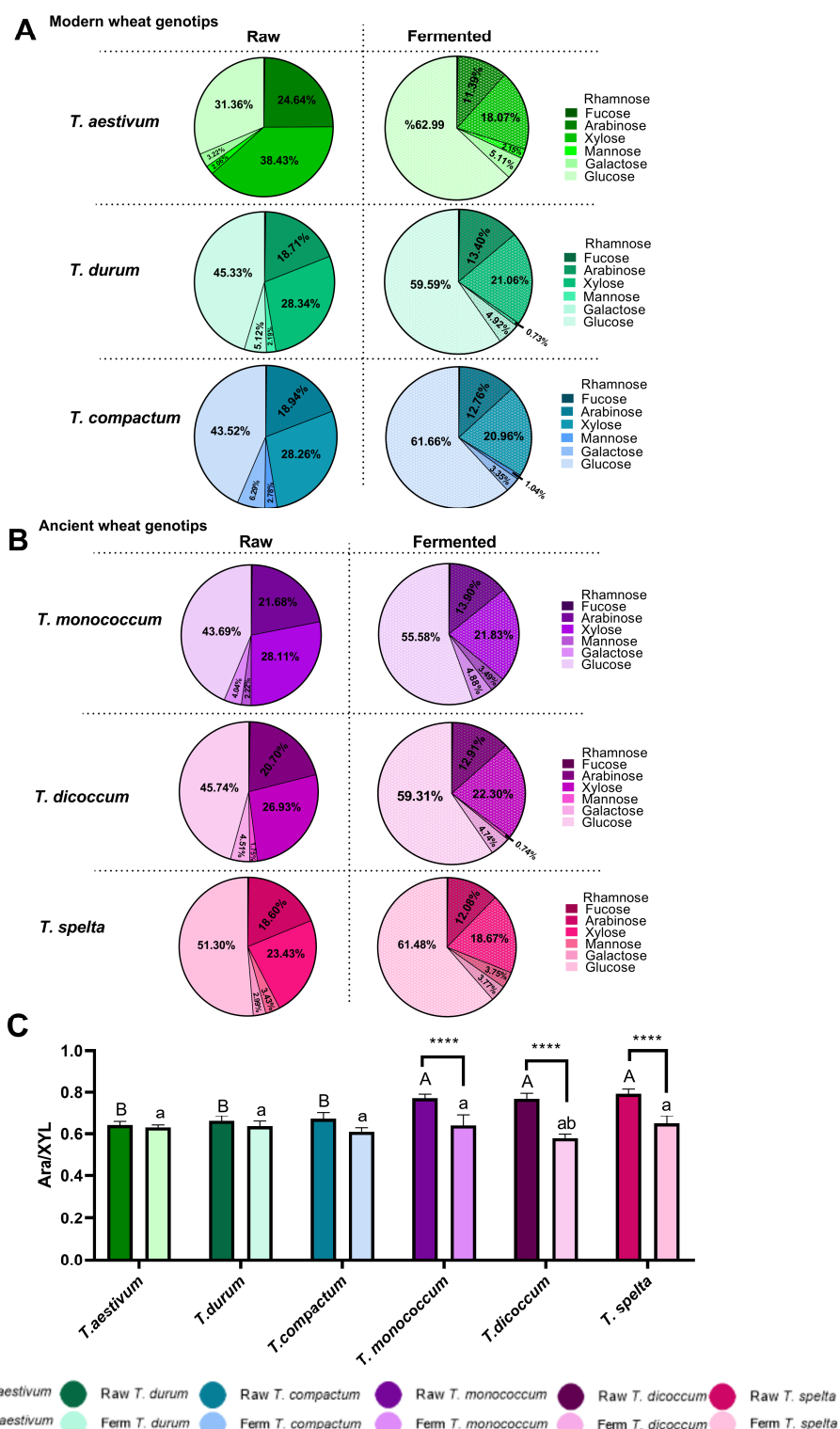


Figure 4. Monosaccharide composition of digestion-derived residues obtained after in vitro digestion. (A) Modern wheat genotypes; (B) ancient wheat genotypes; and (C) arabinose-to-xylose (Ara/Xyl) ratio of raw and fermented wheat samples. Values are expressed as mean $\pm$ SD ($n = 3$). In panel (C), uppercase letters indicate statistically significant differences among raw samples across wheat genotypes, whereas lowercase letters indicate statistically significant differences among fermented samples across wheat genotypes. Asterisks indicate significant differences between raw and fermented samples across wheat genotype (**** $p < 0.0001$). Multiple comparisons were performed using Tukey's multiple comparison test following two-way ANOVA. Results of the two-way ANOVA evaluating the main effects of genotype, pre-fermentation, and their interaction are provided in Supplementary Table S1.

The reduction in the Ara/Xyl ratio following pre-fermentation was supported by significant main effects of genotype and pre-fermentation and a significant genotype $\times$ pre-fermentation interaction (Supplementary Table S1), indicating that the magnitude of this response differed among wheat genotypes. Given the analytical limitations described above, this reduction should be interpreted as a compositional change rather than direct evidence of arabinoxylan debranching or structural reorganization. Arabinoxylan and xylan degradation requires the action of hemicellulolytic enzymes, including xylanases, β -xylosidases, and α -L-arabinofuranosidases. However, *S. cerevisiae* has limited intrinsic capacity to degrade arabinoxylan and xylan, although several studies have introduced heterologous hemicellulolytic enzymes or engineered enzyme systems in *Saccharomyces cerevisiae* to enable or enhance xylan hydrolysis and utilization [48–53]. Therefore, the observed decrease in the Ara/Xyl ratio in the present study should be interpreted as a fermentation-associated compositional change rather than as direct evidence of enzymatic arabinoxylan degradation by baker's yeast. From a functional perspective, the observed compositional changes may influence the availability of carbohydrate substrates during colonic fermentation. Monosaccharide composition and dietary fiber characteristics are recognized as important factors affecting microbial substrate utilization and fermentation behavior [54–57]. Accordingly, these compositional differences may be relevant to the microbial and SCFA responses observed during subsequent fecal fermentation [54–58].

Although all wheat genotypes showed a reduction in the Ara/Xyl ratio following pre-fermentation, the magnitude of this response differed among genotypes, consistent with the significant genotype $\times$ pre-fermentation interaction (Supplementary Table S1). Such variation may reflect genotype-related differences in dietary fiber composition and arabinoxylan characteristics reported previously [12,59,60].

3.5. Microbial Community Structure and Diversity

Comparisons of the overall structures of fecal microbiota communities before and after fermentation were performed using Bray–Curtis dissimilarity based on the relative abundance of OTUs at 97% similarity (Figure 5A). The first two principal coordinate axes accounted for 51.88% of the total variation (PCo1 = 36.55% and PCo2 = 15.33%). No significant differences were observed among the initial, blank (post-fermentation), and inulin (post-fermentation) samples ($p > 0.05$, AMOVA), indicating that these groups exhibited comparable overall microbial community structures. In contrast, significant differences in community structure were observed between the raw and pre-fermented wheat samples (post-fermentation) and the initial, blank, and inulin samples ($p < 0.001$, AMOVA), indicating that wheat substrates induced distinct shifts in the overall fecal microbial community structure. Notably, these shifts were observed following fermentation of both raw and pre-fermented wheat, but not inulin. This difference may reflect the greater structural and compositional complexity of wheat-derived substrates, which may provide a broader range of fermentable substrates and thereby support the growth of a wider range of microbial taxa. In contrast, the relatively simpler composition of inulin may favor a narrower subset of microorganisms, resulting in less pronounced changes in overall community structure. Furthermore, a significant difference in Bray–Curtis-based community structure was observed between raw and pre-fermented wheat samples ($p < 0.001$, AMOVA), indicating that pre-fermentation altered the microbial community response to wheat-derived substrates during colonic fermentation.

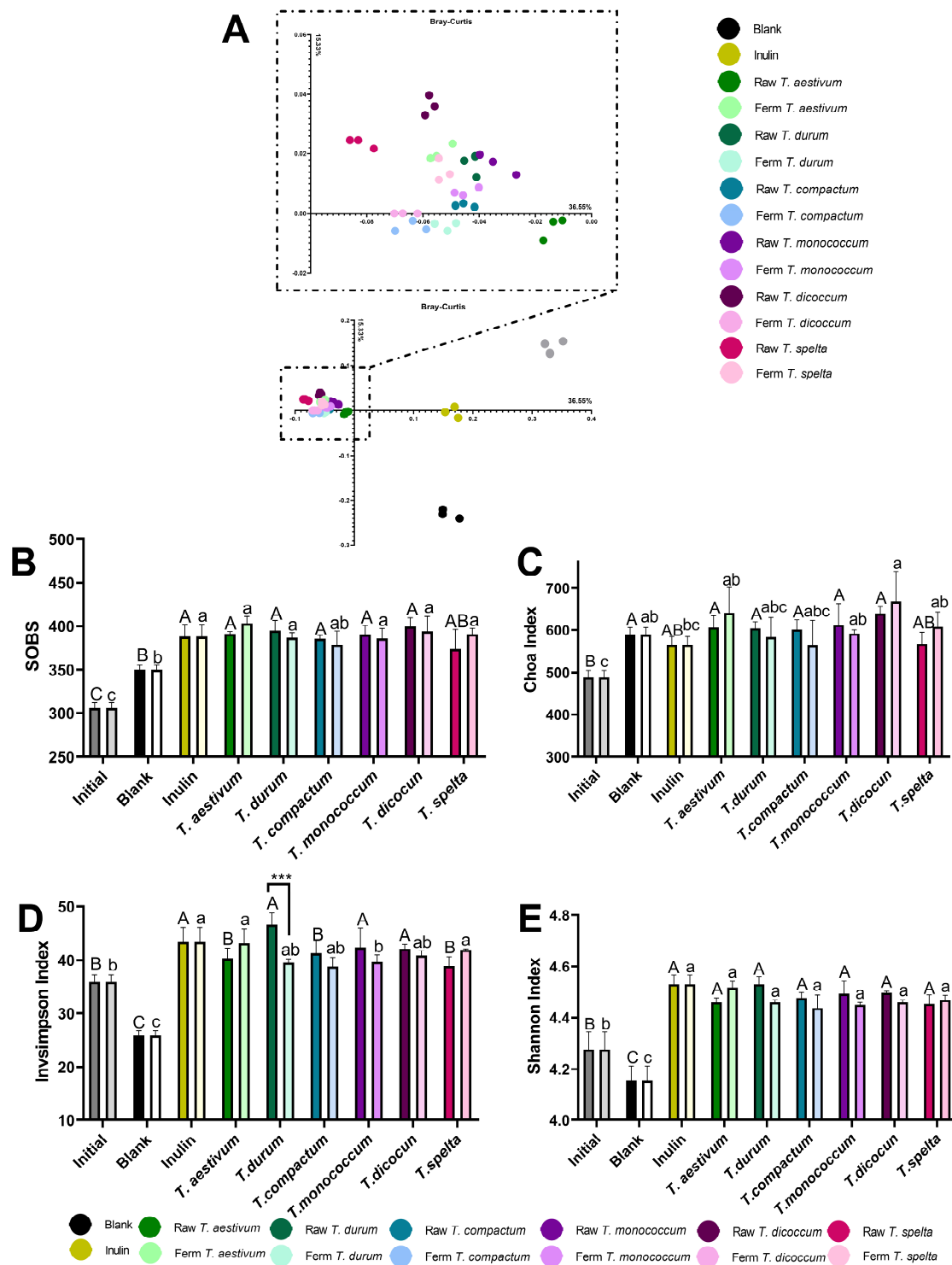


Figure 5. Effects of digestion-derived wheat residues on gut microbial community structure and diversity. (A) Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity showing β -diversity of microbial communities; (B) observed species; (C) Chao index; (D) inverse Simpson index; and (E) Shannon index representing α -diversity. Values in panels (B–E) are expressed as mean $\pm$ SD ($n = 3$ technical/process fermentation replicates using a pooled fecal inoculum). Uppercase letters indicate statistically significant differences among raw samples across wheat genotypes, whereas lowercase letters indicate statistically significant differences among fermented samples across wheat genotypes. Asterisks indicate significant differences between raw and fermented samples within each wheat genotype ($*** p < 0.001$). Multiple comparisons were performed using Tukey’s multiple comparison test following two-way ANOVA. Results of the two-way ANOVA evaluating the main effects of genotype, pre-fermentation, and their interaction on α -diversity indices are provided in Supplementary Table S1.

Alpha diversity was evaluated using the number of observed species (Figure 5B), Chao index (Figure 5C), inverse Simpson index (Figure 5D), and Shannon index (Figure 5E). Observed species and Chao values were generally higher in wheat and inulin treatments than in the initial inoculum and blank, indicating greater microbial richness after substrate fermentation. However, no consistent effect of pre-fermentation was observed across all α -diversity indices. Two-way ANOVA identified significant genotype $\times$ pre-fermentation interactions for the inverse Simpson and Shannon indices, together with a significant main effect of pre-fermentation on the Shannon index (Supplementary Table S1). Similarly, Demuth et al. [44] reported that bacterial α -diversity remained largely unchanged following in vitro fermentation of arabinoxylan fractions despite differences in processing conditions. Inverse Simpson and Shannon indices varied relatively little across most wheat samples, indicating limited changes in overall α -diversity. Thus, pre-fermentation had an index-dependent effect on α -diversity rather than producing a consistent shift across all diversity measures. Because fecal samples from three donors were pooled prior to fermentation, these diversity analyses describe the response of the pooled microbial community and do not represent inter-individual donor variability.

3.6. Changes in Selected Microbial Taxa

To evaluate associations between the compositional characteristics of the wheat residues and microbial community responses, the impacts of wheat genotype and pre-fermentation on fecal microbiota composition were assessed using OTU-level taxonomic analysis. Fold changes relative to the initial inoculum in the top 50 most abundant OTUs after 24 h of in vitro fecal fermentation are shown in Figure 6A. The heatmap provides an overview of OTU response patterns across treatments and was not intended as a formal differential-abundance analysis. Statistical analyses of the five representative OTUs were performed using two-way ANOVA with genotype and pre-fermentation as fixed factors, followed by Tukey's multiple comparison test. The main effects of genotype and pre-fermentation and their interaction are summarized in Supplementary Table S1, whereas relative abundances and post hoc comparisons are presented in Figure 6B.

Overall, the heatmap indicated treatment-associated variation in specific microbial taxa rather than a uniform community-wide response. The heatmap suggested that OTU2 (*Parabacteroides distasonis*) showed higher relative abundance in several wheat treatments than in the initial inoculum and blank. Two-way ANOVA showed a significant main effect of genotype ($p < 0.0001$) and a significant genotype $\times$ pre-fermentation interaction ($p = 0.0493$), whereas the main effect of pre-fermentation was not significant (Supplementary Table S1). The significant interaction indicates that the response to pre-fermentation varied among wheat genotypes. This response is consistent with the reported carbohydrate-utilization capacity of *P. distasonis* [61]. The response of saccharolytic taxa such as *Parabacteroides* and *Kineothrix* may be associated with differences in the composition of the recovered digestion-derived residues described in Section 3.4; however, the present study does not establish a causal relationship between residue composition and taxon-level responses.

OTU16 (*Butyrivibrio crossotus*) also showed higher relative abundance in selected wheat treatments. Statistical analysis showed significant main effects of genotype and pre-fermentation, together with a significant genotype $\times$ pre-fermentation interaction (all $p < 0.0001$; Supplementary Table S1), indicating that the response to pre-fermentation differed among wheat genotypes. Given the association of *Butyrivibrio* with butyrate production [62], the observed response may be relevant to the SCFA patterns (reported in Section 3.7). This response may also be associated with differences in the monosaccharide composition of the recovered digestion-derived residues described in Section 3.4;

however, direct relationships between residue composition and microbial responses were not evaluated.

OTU12 (*Kineothrix*) showed higher relative abundance in most wheat treatments than in the initial, blank, and inulin controls. Two-way ANOVA showed a significant genotype effect ($p < 0.0001$) and genotype $\times$ pre-fermentation interaction ($p = 0.0012$), whereas the main effect of pre-fermentation was not significant (Supplementary Table S1). *Kineothrix alysoides* has been described as a saccharolytic, butyrate-producing bacterium, supporting the potential functional relevance of this response [63].

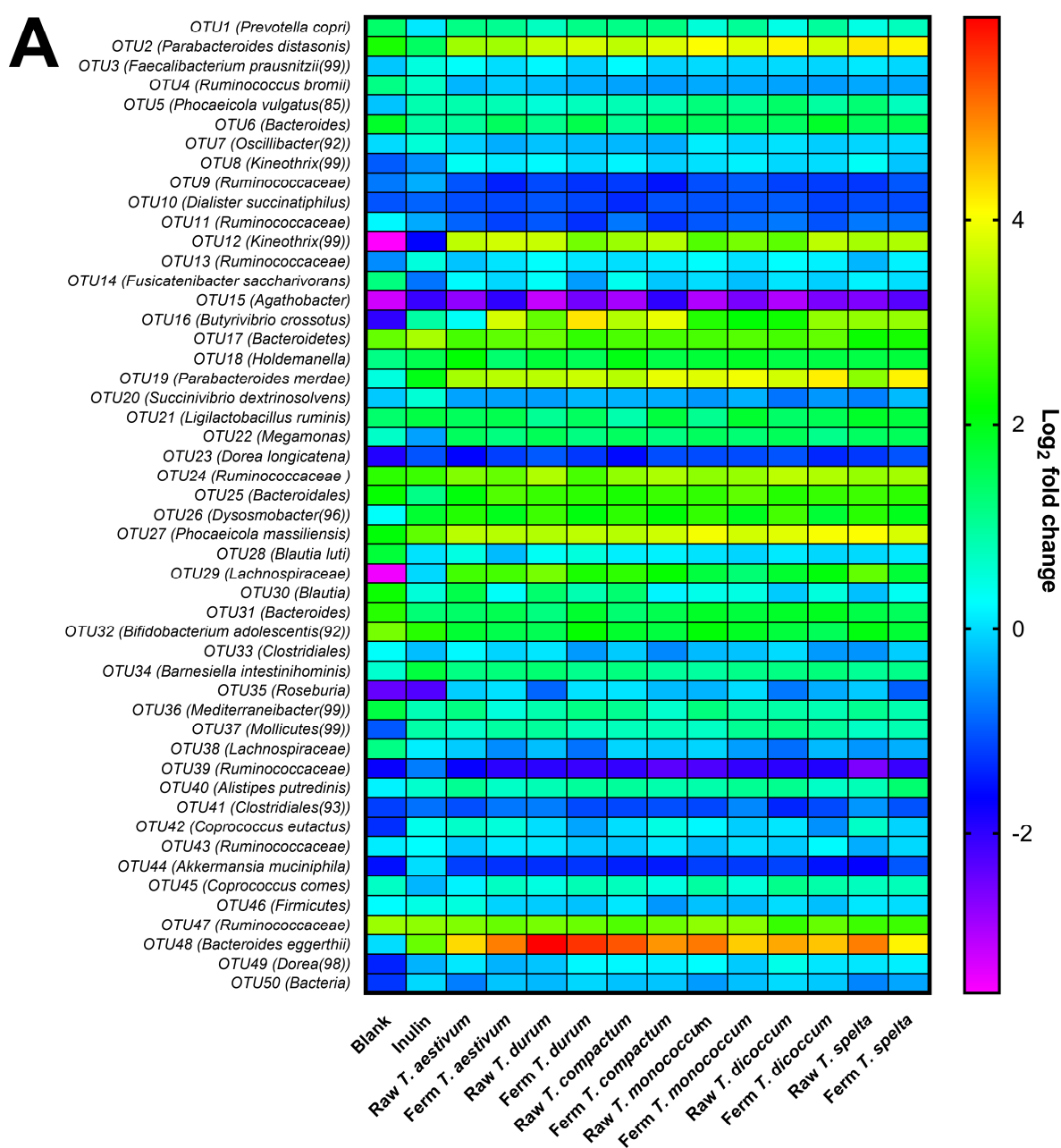


Figure 6. Cont.

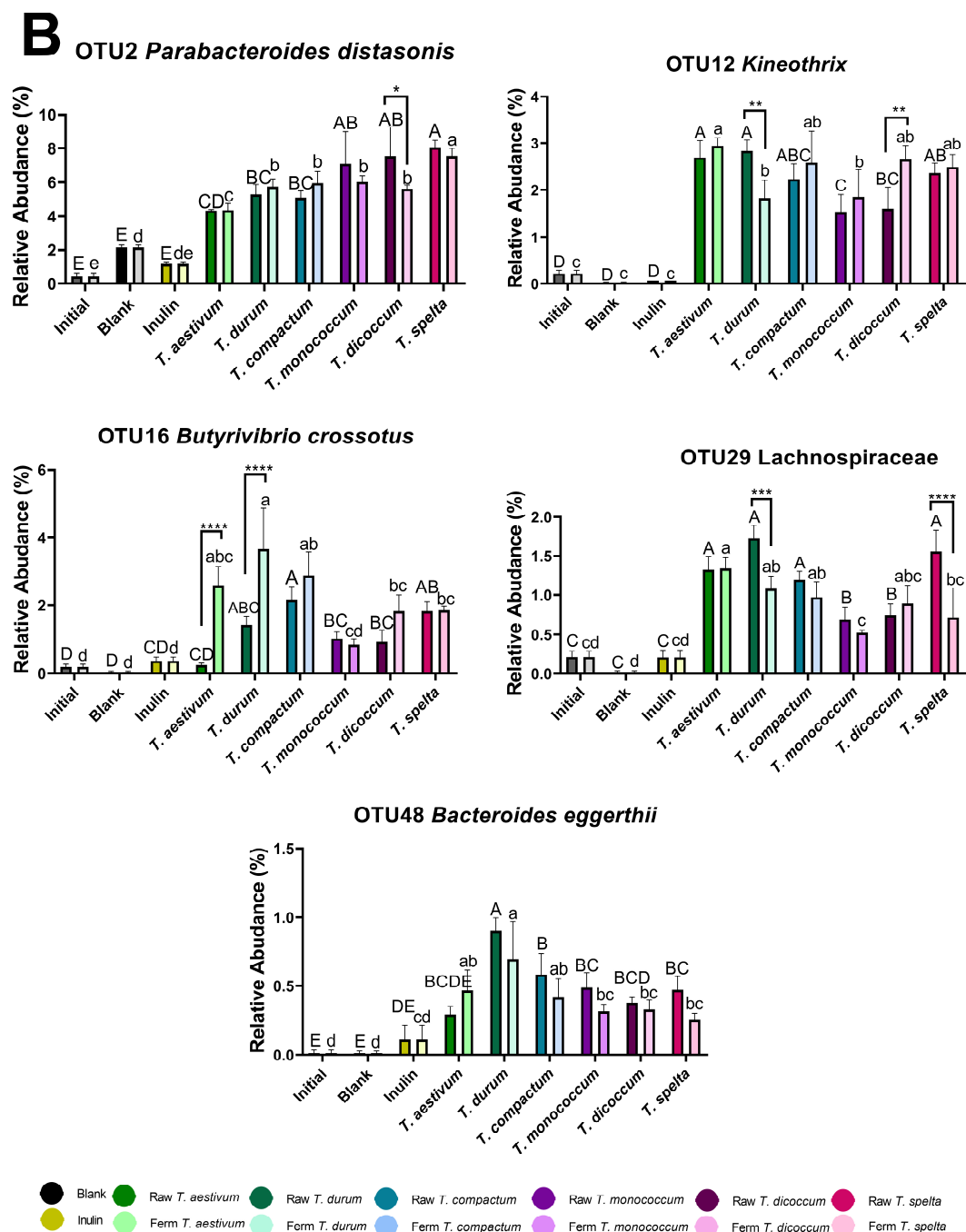


Figure 6. Changes in selected microbial taxa after 24 h of in vitro fecal fermentation. (A) Heatmap showing the log₂ fold changes of the top 50 most abundant OTUs relative to the initial inoculum. Fold changes were calculated from OTU relative abundances. Warmer colors indicate higher relative abundance, whereas cooler colors indicate lower relative abundance compared with the initial inoculum. The heatmap provides a descriptive overview of taxon-level response patterns and does not represent statistical significance. (B) Relative abundances of five representative OTUs: OTU2 (*Parabacteroides distasonis*), OTU12 (*Kineothrix*), OTU16 (*Butyrivibrio crossotus*), OTU29 (*Lachnospiraceae*), and OTU48 (*Bacteroides eggerthii*). Values are expressed as mean $\pm$ SD ($n = 3$ technical/process fermentation replicates using a pooled fecal inoculum). Uppercase letters indicate statistically significant differences among raw samples across wheat genotypes, whereas lowercase letters indicate statistically significant differences among fermented samples across wheat genotypes. Asterisks indicate significant differences between raw and fermented samples within each wheat genotype (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$). Multiple comparisons were performed using Tukey's multiple comparison test following two-way ANOVA. Results of the two-way ANOVA evaluating the main effects of genotype, pre-fermentation, and their interaction are provided in Supplementary Table S1.

Lachnospiraceae-related OTUs, particularly OTU29, showed notable but variable responses across wheat treatments. Two-way ANOVA showed significant effects of genotype, pre-fermentation, and their interaction (all $p \leq 0.0002$; Supplementary Table S1), indicating that the response to pre-fermentation differed among wheat genotypes. Lachnospiraceae are widely recognized as fiber-fermenting bacteria involved in SCFA production, although their functional roles can be species- and context-dependent [64,65]. OTU48 (*Bacteroides eggerthii*) also showed marked variation among treatments. For this OTU, two-way ANOVA identified significant main effects of genotype ($p < 0.0001$) and pre-fermentation ($p = 0.0201$), whereas the genotype $\times$ pre-fermentation interaction was not statistically significant ($p = 0.0529$; Supplementary Table S1). Thus, unlike OTU2, OTU12, OTU16, and OTU29, the response of OTU48 to pre-fermentation was not demonstrably genotype-dependent. This pattern is biologically plausible because *Bacteroides* species are highly adapted to utilize diverse dietary polysaccharides through polysaccharide utilization systems [66,67].

The effects of pre-fermentation were OTU- and genotype-dependent rather than uniformly stimulatory. Some raw wheat samples showed higher abundances of selected OTUs than their fermented counterparts. These differences may reflect processing-associated changes in the recovered digestion-derived residues [44,68]; however, molecular-weight distribution, arabinoxylan solubility, and arabinoxylan-derived oligosaccharides were not determined in the present study, and the underlying mechanisms therefore remain hypothetical.

Overall, pre-fermentation did not exert a uniform effect across the selected microbial taxa. Genotypes significantly influenced all five representative OTUs, whereas the effect of pre-fermentation varied among taxa. Significant genotype $\times$ pre-fermentation interactions were observed for OTU2, OTU12, OTU16, and OTU29, but not OTU48 (Supplementary Table S1), demonstrating taxon-specific and, for most representative OTUs, genotype-dependent responses to pre-fermentation. These responses may be associated with compositional differences among the recovered digestion-derived residues; however, as residual yeast-derived carbohydrates were not quantified separately and no correlation or substrate-utilization analyses were performed, these relationships should be interpreted as associations rather than direct causal mechanisms.

3.7. Short-Chain Fatty Acid Production During In Vitro Fermentation

Microbial SCFAs, including acetate, propionate, and butyrate, were quantified throughout in vitro fermentation, and total SCFA values were calculated as the sum of these metabolites at each time point (Figure 7). Inulin, used as a rapidly fermentable positive control, produced higher total SCFA levels at early fermentation stages, consistent with its rapid utilization by the gut microbiota [69]. In contrast, the wheat samples showed slower SCFA accumulation, indicating more gradual fermentation kinetics, consistent with previous in vitro fermentation studies of wheat-derived and slowly fermentable dietary fiber substrates [70,71]. SCFA concentrations increased progressively in all wheat samples over time, and by 48 h, several wheat genotypes reached total SCFA concentrations of approximately 60–70 mM, compared with approximately 90 mM for inulin. The taxon-level responses observed in Section 3.6 were accompanied by the SCFA production patterns reported here. Overall, the wheat residues supported substantial SCFA production during prolonged fermentation [72].

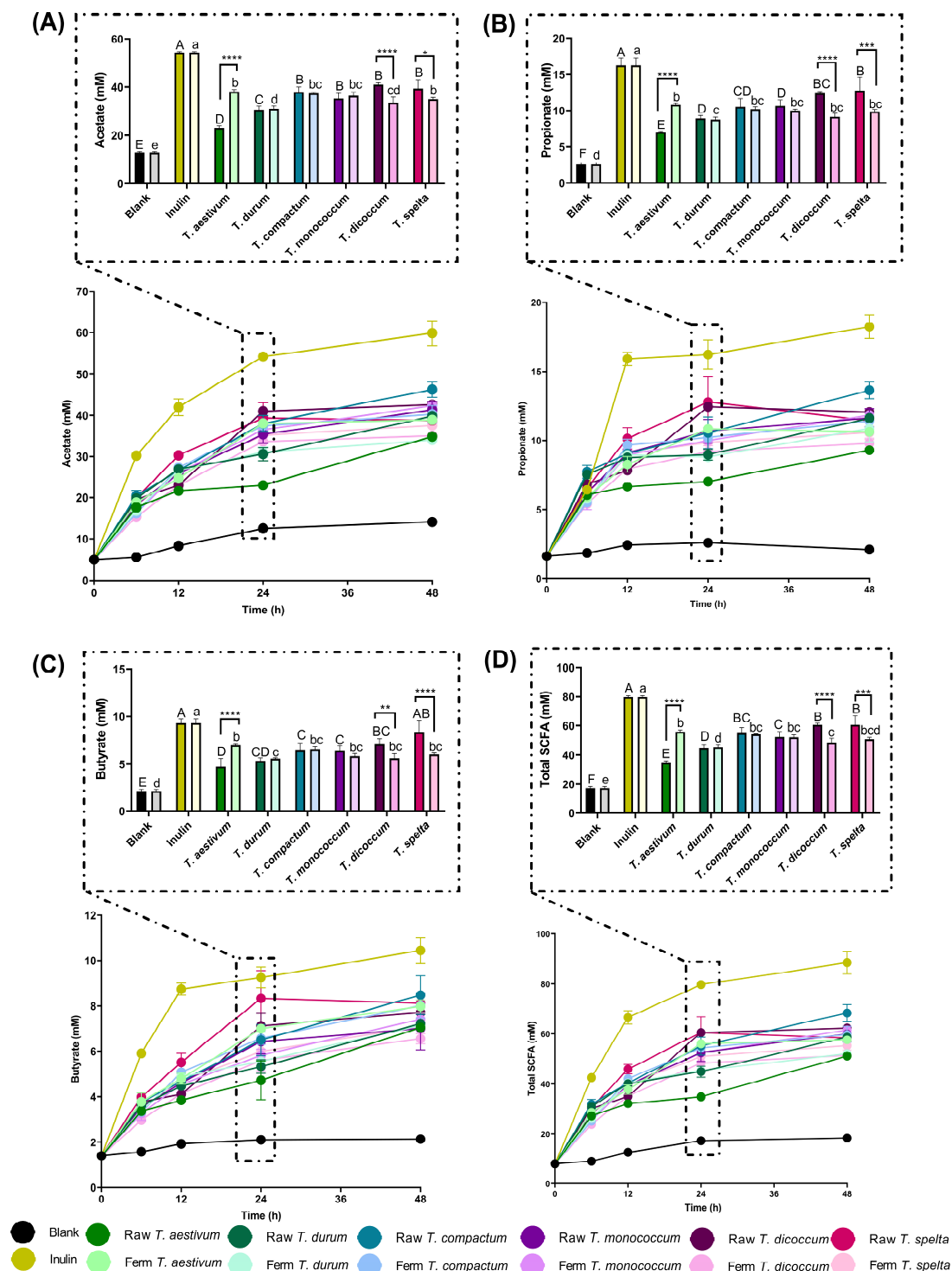


Figure 7. SCFA production during in vitro fecal fermentation of raw and fermented wheat samples. (A) Acetate concentration; (B) propionate concentration; (C) butyrate concentration; and (D) total SCFA concentration. Values are expressed as mean $\pm$ standard deviation ($n = 3$ technical/process fermentation replicates using a pooled fecal inoculum). Insets show statistical comparisons at 24 h. Uppercase letters indicate statistically significant differences among raw samples across wheat genotypes, whereas lowercase letters indicate statistically significant differences among fermented samples across wheat genotypes. Asterisks indicate significant differences between raw and fermented samples within each wheat genotype (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$). Multiple comparisons were performed using Tukey's multiple comparison test following two-way ANOVA. Results of the two-way ANOVA evaluating the main effects of genotype, pre-fermentation, and their interaction at 24 h are provided in Supplementary Table S1.

At 24 h, two-way ANOVA revealed significant effects of wheat genotype on acetate, propionate, butyrate, and total SCFA production ($p < 0.0001$; Supplementary Table S1). Significant genotype $\times$ pre-fermentation interactions were also observed for all SCFA outcomes ($p < 0.0001$), indicating that the effect of pre-fermentation on SCFA production at 24 h differed among wheat genotypes. In contrast, the main effect of pre-fermentation was not significant for any SCFA outcome ($p > 0.05$). Thus, the magnitude and direction of differences between raw and fermented samples varied among wheat genotypes and SCFA fractions. These genotype-dependent responses may be associated with compositional differences among the recovered digestion-derived residues. However, because structural characteristics of the dietary fiber fractions were not directly determined, the compositional basis of these differences cannot be established from the present data.

Acetate was the dominant SCFA across treatments, whereas propionate and butyrate accumulated progressively over time. This pattern is consistent with acetate being a major product of microbial carbohydrate fermentation and with its potential involvement in cross-feeding interactions supporting butyrate production. The gradual accumulation of butyrate may therefore reflect secondary fermentation processes and microbial cross-feeding interactions [72,73].

The significant genotype $\times$ pre-fermentation interactions at 24 h indicate that pre-fermentation did not exert a uniform effect on SCFA production across wheat genotypes. Because a yeast-only process control was not included and residual yeast-derived carbohydrates were not quantified, the relative contributions of wheat- and yeast-derived carbohydrates to these responses cannot be distinguished. Furthermore, molecular-weight distribution, arabinoxylan solubility, oligosaccharide formation, and other structural characteristics were not determined; therefore, the mechanisms underlying these differences remain hypothetical. Taken together, these results show progressive accumulation of SCFAs over the 48-h fermentation period, while the 24-h factorial analysis revealed genotype-dependent responses to pre-fermentation. Compared with the rapidly fermentable inulin control, the recovered digestion-derived wheat residues exhibited a more gradual SCFA accumulation pattern, consistent with differences in fermentation kinetics between these substrates.

4. Conclusions

This study demonstrated that yeast-mediated pre-fermentation was associated with changes in the composition of recovered digestion-derived wheat residues and with differences in subsequent in vitro gut microbial fermentation. Pre-fermentation increased Folin–Ciocalteu-reactive values and antioxidant capacity and was associated with lower residual starch and protein contents following upper gastrointestinal digestion. Monosaccharide analysis showed lower relative proportions of arabinose and xylose and a higher relative proportion of glucose in the pre-fermented wheat samples, with the magnitude of these changes differing among wheat genotypes. In vitro fecal fermentation resulted in significant shifts in the microbial community structure of pre-fermented wheat samples compared with raw wheat, as demonstrated by Bray–Curtis dissimilarities ($p < 0.001$, AMOVA), whereas α -diversity responses were limited. Selected carbohydrate-fermenting and SCFA-producing taxa, including *Parabacteroides distasonis*, *Butyrivibrio crossotus*, and *Kineothrix*, showed taxon- and genotype-dependent responses. All wheat samples supported SCFA production, with pre-fermented wheat samples exhibiting a more gradual SCFA accumulation pattern than the rapidly fermentable inulin control. Taken together, these findings suggest that yeast-mediated pre-fermentation can modify wheat composition and, in turn, influence fecal microbial community structure and fermentation dynamics, with genotype-dependent responses.

Several limitations of the present study should be considered when interpreting these findings. First, the pre-fermentation model was designed as a simplified laboratory system rather than a bread-making process. Consequently, the findings should not be interpreted as directly representing baked wheat products, since dough development, proofing, baking, starch gelatinization, protein denaturation, and other technological processes may further modify substrate composition and subsequent microbial fermentation. In addition, because untreated wheat flour was used as the control and a non-inoculated processed control was not included, the individual contributions of hydration, incubation, endogenous wheat enzymatic activity, drying, and yeast fermentation could not be separated. Furthermore, the absence of a yeast-only control prevented discrimination between wheat- and yeast-derived contributions to the recovered carbohydrate fractions. Future studies should determine whether similar responses are observed in finished cereal products such as bread and should include yeast-only and non-inoculated processed controls to distinguish the specific contributions of yeast biomass and processing effects.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation12090427/s1>, Table S1: Two-way ANOVA results for the main effects of wheat genotype and pre-fermentation and their interaction on proximate composition, antioxidant properties, post-digestion composition, monosaccharide profiles, selected microbial taxa, and SCFA.

Author Contributions: Conceptualization, Y.E.T.; methodology, T.C.K. and Y.E.T.; validation, T.C.K. and Y.E.T.; formal analysis, İ.S., T.C.K., Y.E.T., M.A.A.G. and S.R.L.; investigation, T.C.K., İ.S., M.A.A.G. and Y.E.T.; resources, S.R.L. and Y.E.T.; writing—original draft preparation, T.C.K. and Y.E.T.; writing—review and editing, T.C.K., M.A.A.G., S.R.L. and Y.E.T.; visualization, T.C.K. and Y.E.T.; supervision, Y.E.T.; project administration, Y.E.T.; funding acquisition, S.R.L. and Y.E.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Health Sciences Scientific Research Ethics Committee of Necmettin Erbakan University (approval number: 14580; date of approval: 7 June 2023).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data will be made available on request.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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