

Development of nutritionally enhanced sourdough bread through *Tritordeum* bran incorporation and assessment in an *in vitro* gut simulation

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ABSTRACT

This study for the first time investigated how *Tritordeum* bran incorporation and starter-assisted sourdough fermentation influence bread nutritional features and gut microbiota, using a defined starter composed of *Lactiplantibacillus plantarum* LM108.5, *Pediococcus pentosaceus* TLD10–13, *Lactobacillus curvatus* TLD10–17, and *Saccharomyces cerevisiae* TYD10–5. The bran-based sourdough bread (BSD-B3) showed higher total phenolic compounds (1185 mg GAE/kg), DPPH radical scavenging activity (10.83 mM BHT/100 g), β -glucans (0.39 %), arabinoxylans (3.22 %), and total free amino acids (1758 mg/kg), together with a reduced predicted glycemic index (8.83 %) and improved *in vitro* protein digestibility (up to 77.2 %) compared to the control (baker's yeast bread (BYB)). Targeted phenolic profiling revealed increased levels of protocatechuic, ferulic, and vanillic acids in BSD-B3, highlighting the contribution of bran and sourdough fermentation to phenolic bioavailability. The impact of *Tritordeum* sourdough breads with or without bran (TSD-B1 and BSD-B3) on colonic microbiota was evaluated *in vitro* using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME[®]). Application of sourdough bread digesta in the SHIME[®] model enhanced short-chain fatty acid production, with BSD-B3 sustaining higher butyrate levels, while TSD-B1 and BYB favored acetate- and propionate-linked bifidogenic activity. Amplicon sequencing (16S rRNA gene) indicated that these metabolic shifts were accompanied by distinct changes in microbial composition and diversity. Our findings suggest that sourdough fermentation, particularly when combined with *Tritordeum* bran incorporation, produced nutritionally superior bread that selectively alters the gut microbiota.

1. Introduction

In recent years, there has been a growing interest in alternative cereals that offer sustainable solutions to meet the demands of a growing population and reduce dependence on traditional wheat. *Tritordeum*, an amphidiploid cereal resulting from the cross between the South American wild barley (*Hordeum chilense*) and cultivated durum wheat (*Triticum turgidum* ssp. *durum*), has emerged as a promising candidate (Arora et al., 2022). Primarily grown in the Mediterranean region, *Tritordeum* combines the nutritional characteristics of durum wheat and barley, providing an excellent nutritional profile rich in soluble and insoluble dietary fibers (DF) (Suchowilska et al., 2021).

Dietary fiber plays a crucial role in promoting health and preventing

chronic diseases. Adequate intake of DF has been associated with a reduced risk of type 2 diabetes, obesity, certain cancers, and cardiovascular diseases (Kuznesof et al., 2012; Li and Ma, 2024; Veronese et al., 2025). The World Health Organization recommends a daily DF intake ranging from 20 to 45 g, depending on regional dietary habits (Jama et al., 2024; Stephen et al., 2017). The health benefits of DF are attributed to its involvement in weight regulation, lipid metabolism, metabolic improvement, and blood pressure modulation (Pontonio et al., 2020; Sharma et al., 2025). Bran, the outer layer of cereal grains, is a by-product of the milling process and is rich in fibers, such as cellulose, hemicellulose, and lignin. It also contains antioxidant compounds, such as phenolic compounds, carotenoids, and anthocyanins, along with vitamins, minerals, and proteins (Akin et al., 2025; Qin et al., 2025).

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Despite its high nutritional value, the direct incorporation of bran into bakery products poses technological challenges. Bran addition can negatively impact dough processing and bread properties, leading to increased dough stickiness, reduced loaf volume, coarser and darker crumb texture, decreased crumb softness, and loss of crust crispiness. These changes often result in lower consumer acceptability of DF-enriched breads (Aluthge et al., 2025; Hassan et al., 2025).

To address these challenges, sourdough fermentation is widely recognized as a sustainable and promising strategy to improve the techno-functionality of fiber-rich cereal ingredients. Sourdough fermentation offers both technological and environmental advantages. Its sustainability derives from the use of natural microbial consortia, reduced reliance on chemical improvers, and lower energy and water inputs during processing, while its technological value lies in the ability to valorize cereal by-products by improving their digestibility and extending product shelf life (Cai et al., 2024; Gobetti et al., 2014). Starter-assisted sourdough fermentation has been shown to enhance bread quality compared with spontaneous fermentation, primarily by providing better control of key parameters (temperature, inoculum size, pH), which ensures more predictable microbial performance and improved dough structure and texture (Siepmann et al., 2018). Selected lactic acid bacteria (LAB) starters have been successfully used to ferment wheat and rye bran, aiming to enhance technological, physicochemical, nutritional, and sensory properties, and to degrade anti-nutritional factors in leavened baked goods (Coda et al., 2015).

In parallel, the use of environmentally resilient raw materials such as *Tritordeum* further strengthens the sustainability of sourdough-based systems. This novel cereal is adapted to Mediterranean and semi-arid conditions, requires less irrigation and fertilizer, and maintains yield stability under heat and drought stress, aligning with global strategies for climate-resilient and resource-efficient crop production (Ávila et al., 2021). While spontaneous fermentation of *Tritordeum* flour has been explored for its potential to enhance nutrient bioavailability (Arora et al., 2022), the combined influence of *Tritordeum* bran incorporation and controlled starter-assisted fermentation on nutritional quality and gut microbiota interactions remains unexplored. Therefore, this study investigates the incorporation of *Tritordeum* bran by fermenting a flour-bran blend with selected lactic acid bacteria and yeast strains, focusing on the resulting bread's nutritional quality and its effects on the gut microbiota. To this end, a blend of *Tritordeum* flour and bran was first fermented with a pool of LAB and a yeast strain. Subsequently, *Tritordeum* sourdough breads with and without bran were made and characterized based on key nutritional features. Finally, the impact of these sourdough breads on colonic microbial ecosystems was assessed using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). Recent applications of the SHIME® system to sourdough and cereal-based foods have demonstrated reproducible modulation of colonic short-chain fatty acid (SCFA) profiles and beneficial microbial shifts, confirming its suitability for evaluating gut responses to bread matrices (Mastrodonato et al., 2024). This validated *in vitro* model allows to carry out the investigation by excluding the interference of other factors such as individual dietary habits and host physiological factors (Polo et al., 2023).

2. Materials and methods

2.1. Raw material, microbial inoculum, and sourdough preparation

Tritordeum flour and *Tritordeum* bran were obtained from Intini Foods (Puglia, Italy). A mixed starter culture consisting of *Lactiplantibacillus plantarum* LM108.5, *Pediococcus pentosaceus* TLD10-13, *Lactilactobacillus curvatus* TLD10-17 and *Saccharomyces cerevisiae* TYD10-5, was used for sourdough fermentation. These LAB and yeast strains belong to the Culture Collection of the Free University of Bolzano-Bozen. *P. pentosaceus* TLD10-13, *L. curvatus* TLD10-17, and *S. cerevisiae* TYD10-5 were previously isolated from type I *Tritordeum* sourdough,

while *L. plantarum* LM108.5 was isolated from a wheat sourdough. The cultures were kept as frozen stocks at -80°C in de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, Hampshire, UK) for LAB and in Sabouraud broth (SAB; Oxoid, Basingstoke, Hampshire, UK) with 20 % (w/v) glycerol. Before use, strains were sub-cultured twice (4 %, v/v) at 30°C for 24 h in MRS and SAB broth for LAB and yeast, respectively. Temperature was maintained at $30.0 \pm 0.1^{\circ}\text{C}$ using a calibrated incubator to ensure stable conditions throughout incubation. Then, cell pellets from overnight cultures were collected through centrifugation ($14,534 \times g$, 10 min, 4°C), followed by two washing steps with sterile physiological solution (NaCl; CAS 7647-14-5, $\geq 99.5\%$, Sigma-Aldrich, Steinheim, Germany), and resuspended in the same buffer to obtain a final optical density (OD) of 0.25 ± 0.03 at 620 nm for LAB (ca. 9.0 Log CFU/mL) and 1.0 ± 0.03 at 600 nm for yeast (ca. 7.0 Log CFU/mL). These suspensions were subsequently used as inocula for sourdough preparation, providing final cell densities in the dough of approximately 7.0 log CFU/g for LAB and 5.0 log CFU/g for yeast (Stringari et al., 2024).

Two sourdough prototypes were prepared. The first prototype consisted of 100 % *Tritordeum* flour (TSD), while the second prototype consisted of a blend (1:1) of *Tritordeum* flour and *Tritordeum* bran (BSD). The dough yield was set to 200 (Eq. (1)). For TSD, 100 g of *Tritordeum* flour was mixed with 92 mL of tap water, while for BSD, 50 g of *Tritordeum* flour and 50 g of *Tritordeum* bran were combined with 92 mL of tap water. In both formulations, cell suspensions of LAB and yeast, (corresponding to of ca. 7.0 and 5.0 Log CFU/g respectively) were inoculated and mixed using a continuous high-speed mixer ($60 \times g$) for 5 min (Esmach, Type SPI 30 F, Italy) and the resulting doughs were fermented at $30 \pm 1.0^{\circ}\text{C}$ for 24 h. Then mature sourdough was prepared by two back-slopping steps using an inoculum (25 %, w/w) of initially prepared sourdough and fermented at $30 \pm 1.0^{\circ}\text{C}$ for 8 h. Samples were collected before fermentation (T0, after mixing) and after two back-slopping steps (T8) for subsequent analyses.

$$DY = \frac{W_{\text{dough}}}{W_{\text{flour}}} \times 100 \quad (1)$$

where W_{dough} represents the total dough weight (g), including all ingredients (flour, water, and inoculum), and W_{flour} denotes the total flour weight (g).

2.2. Microbial characterization of sourdough

To determine the cell densities of LAB and yeast, 10 g of sample at T0 and T8 from both prototypes was homogenized with 90 mL of sterile physiological solution (NaCl; CAS 7647-14-5, $\geq 99.5\%$, Sigma-Aldrich, Steinheim, Germany) to obtain a 1:10 dilution. Serial decimal dilutions were subsequently prepared using 9 mL of sterile physiological solution. LAB enumeration was under anaerobic conditions on modified MRS (mMRS) agar (Oxoid, Basingstoke, Hampshire, UK), which was supplemented with 0.5 % (w/v) maltose (CAS 6363-53-7, $\geq 99\%$, Sigma-Aldrich, Steinheim, Germany) and 0.5 % (w/v) yeast extract (CAS 8013-01-2, Sigma-Aldrich, Steinheim, Germany), in addition to cycloheximide (0.1 g/L) (CAS 66-81-9, Sigma-Aldrich, Steinheim, Germany). Plates were incubated at $30 \pm 1.0^{\circ}\text{C}$ for 48 h before colony counting. Yeast cell density was determined on Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, Hampshire, UK), supplemented with chloramphenicol (0.1 g/L) (CAS 56-75-7, Sigma-Aldrich, Steinheim, Germany). Plates were incubated at $30 \pm 1.0^{\circ}\text{C}$ for 48 h.

2.3. Physico-chemical, biochemical, and nutritional characterization of sourdough

The pH was measured in both samples (T0 and T8) using a pH meter (Model 507, Crison, Milan, Italy) with a food penetration probe. Total titratable acidity (TTA) was determined after the homogenization of 10

g of sourdough with 90 mL of distilled water and expressed as the amount (mL) of 0.1 M sodium hydroxide (NaOH, $\geq 98\%$, CAS 1310-73-2, Sigma-Aldrich, Steinheim, Germany) required to neutralize the pH until 8.30 units. Water soluble extracts (WSE) were prepared according to Weiss et al. (1993) for the determination of organic acids (lactic and acetic acid), carbohydrates (glucose, fructose and maltose) and ethanol by high-performance liquid chromatography (HPLC), equipped with an Aminex HPX-87H column (300 \times 7.8 mm, ion exclusion; Bio-Rad, Richmond, CA, United States). Elution was done at 70 °C, with a flow rate of 0.6 mL/min using 5 mM sulfuric acid (H₂SO₄; CAS 7664-93-9, 98 %, Sigma-Aldrich, Steinheim, Germany) as mobile phase. A UV detector operating at 210 nm was used for the determination of organic acids, while a refractive index detector (PerkinElmer 200a, Waltham, MA, USA) was used to quantify carbohydrates and ethanol (Tlais et al., 2020). Calibration curves were prepared using analytical standards of lactic acid (CAS 50-21-5, $\geq 98\%$), acetic acid (CAS 64-19-7, $\geq 99.8\%$), glucose (CAS 50-99-7, $\geq 99.5\%$), fructose (CAS 57-48-7, $\geq 99\%$), maltose (CAS 6363-53-7, $\geq 99\%$), and ethanol (CAS 64-17-5, $\geq 99.8\%$) (all standards were purchased from Sigma-Aldrich, Steinheim, Germany). The fermentation quotient (FQ) was determined as the molar ratio between lactic and acetic acids.

2.3.1. Dietary fibers

Soluble (SDF) and insoluble (IDF) dietary fiber were determined using the AOAC enzymatic-gravimetric methods 993.19 and 985.29, respectively. Total dietary fiber (TDF) was calculated as the sum of IDF and SDF.

2.3.2. Free phenolic compounds profiling

To evaluate the impact of fermentation and bran incorporation, the free phenolic compounds were evaluated in methanol/water-soluble extract (MWSE) of freeze-dried dough samples of TSD and BSD at T0 and T8. Briefly, each sample (2 g) was mixed with 8 mL of 80 % methanol (CH₃OH; CAS 67-56-1, $\geq 99.9\%$, Sigma-Aldrich, Steinheim, Germany) and then mixture was acidified with 0.1 % hydrochloric acid (HCl; CAS 7647-01-0, 37 %, Sigma-Aldrich, Steinheim, Germany). Then this mixture was subjected to sonication (amplitude 60) using a microprobe (Vibra-Cell sonicator; VCX series, Sonics & Materials Inc., Danbury, CT, USA) for 1 min (2 cycles, 30 s/cycle, 5 min interval between cycles). Subsequently, the mixture was continuously stirred for 1 h, followed by centrifugation at 14,534 \times g for 10 min to separate the supernatant from the solid phase. Resulting supernatant was filtered (0.22 μ m membrane filter, Millipore Corporation, USA) before analysis. LC-MS platform composed of an Ultimate 3000 RSLCnano system, coupled to a Q-Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was employed for free phenolic compounds determination.

Chromatographic separation of phenolic compounds was performed through a Waters Acquity HSS T3 column (1.8 μ m, 100 mm \times 2.1 mm) (Milford, USA) by using the binary gradient program as follows: 0 min, 2 % B; from 0 to 3 min, linear gradient to 20 % B; from 3 to 4.3 min, isocratic 20 % B; from 4.3 to 9 min, linear gradient to 45 % B; from 9 to 11 min, linear gradient to 100 % B; from 11 to 13 min, wash at 100 % B; from 13.01 to 15 min, and back to the initial conditions of 5 % B (solvent A = water + 0.1 % formic acid (HCOOH; CAS 64-18-6, $\geq 98\%$, Sigma-Aldrich, Steinheim, Germany), while solvent B was acetonitrile (CH₃CN; CAS 75-05-8, $\geq 99.9\%$ purity, Sigma-Aldrich, Steinheim, Germany) + 0.1 % formic acid (CAS 64-18-6, $\geq 98\%$, Sigma-Aldrich, Steinheim, Germany). The flow rate was set at 300 μ L/min, and the temperature of the column was 40 °C. The LC-MS system was equipped with a Higher Collisional Energy Dissociation cell (HCD), and a Heated Electro Spray Ionization (HESI) interface was adopted for LC-HRMS coupling. MS detection occurred in a Targeted-SIM/dd-MS2 mode with a negative polarity, according to the following parameters: spray voltage, 2.80 kV; sheath gas flow rate, at 35 arbitrary units; auxiliary gas flow rate, at 10 arbitrary units; capillary temperature, at 300 °C; S lens RF level, at 50

arbitrary units; capillary gas heater temperature, at 280 °C. The Q-Exactive plus™ mass spectrometer settings were the following: mass scan range, 80–1200 *m/z*; resolution, 70,000 (FWHM); Automatic Gain Control (AGC) Target, 5 \times 10⁶ ions; maximum injection time (IT), 100 ms. Compounds were identified based on their reference standard, retention time, and [*M* – *H*] ions. Peak areas, obtained from extracted Ion Current (XIC) chromatographic traces were used as a measurement of MS response and employed for calibration curve preparation based on commercial standards acids and for the quantification of phenolic compounds in MWSE. Data processing was carried out through the Xcalibur™ software (v. 3.1., Thermo Fisher Scientific, USA) (Shazad et al., 2025).

2.3.3. Antioxidant activity and total phenols

Antioxidant activity and total phenolic content of TSD and BSD at T0 and T8 were determined, according to the methods described by Tlais et al. (2021). The MWSE of the samples were used to determine the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. A DPPH stock solution (0.1 mM in 80 % methanol; CH₃OH, CAS 67-56-1, $\geq 99.9\%$, Sigma-Aldrich, Steinheim, Germany) was freshly prepared and kept in the dark until use. Absorbance was measured at 517 nm against a reagent blank (Tlais et al., 2021). The radical scavenging activity was expressed relative to butylated hydroxytoluene (BHT; CAS 128-37-0, $\geq 99\%$, Sigma-Aldrich, Steinheim, Germany) at a concentration of 75 mg/L, used as the reference antioxidant. The total phenolic content was determined using the Folin-Ciocalteu assay, and the results were expressed as gallic acid equivalents (Tlais et al., 2021).

2.3.4. Anti-nutritional factors (ANF), β -glucan and arabinoxylan (AX) determination

The concentration of ANF such as phytic acid and raffinose series oligosaccharide (RFO) were assessed using the Megazyme kit K-PHYT 05/07 (Megazyme International Ireland limited, Bray, Ireland) and Raffinose/d-Galactose kits (Megazyme International, Ireland) respectively, according to the manufacturer's instructions. The β -glucan content was determined according to AOAC Method 995.16 and AACC Method 32-23, using the Mixed-linkage β -glucan Assay Kit (Megazyme, Bray, Ireland) of freeze-dried samples. Briefly, 80–120 mg of sample was transferred to a glass centrifuge tube. The sample was wet with 0.2 mL of 50 % (v/v) aqueous ethanol, followed by the addition of 4.0 mL of 20 mM sodium phosphate buffer (pH 6.5) and vortex mixing. The mixture was incubated at 100 °C for 2 min with intermittent stirring and then cooled to 50 °C. After adding 0.2 mL of diluted lichenase, the sample was incubated at 50 \pm 1.5 °C for 1 h with regular stirring. Subsequently, 5.0 mL of 200 mM sodium acetate buffer (pH 4.0) was added, and the mixture was centrifuged at 1000 \times g for 10 min. Aliquots (0.1 mL) of the supernatant were treated with 0.1 mL of diluted β -glucosidase (reaction tubes) or 0.1 mL of 50 mM acetate buffer (reaction blank) and incubated at 50 \pm 1.5 °C for 10 min. Finally, 3.0 mL of GOPOD reagent was added, and the tubes were incubated at 50 \pm 1.5 °C for 20 min. The absorbance was measured at 510 nm against the reagent blank, and β -glucan content was calculated based on calibration standards and sample dilution. Similarly, the AX was quantified using a commercial assay kit (K-KYLOSE 10/20 Megazyme, Bray, Ireland) according to the manufacturer's protocol.

2.4. Bread making

Four different breads with DY of 150 were prepared at the Bakery Insperience Pilot Plant of the Micro4Food lab (Free university of Bozen-Bolzano, Italy). In all sourdough-based formulations, the sourdough inoculum was added at 25 % [w/w] of the total dough weight; that is, for every 100 g of dough, 25 g consisted of fermented sourdough. The sourdough inoculum itself was propagated at a DY of 200 and therefore contained equal parts flour and water (50:50, w/w). The remaining flour and tap water were added in amounts that ensured a final DY of 150 in

all bread formulations. The first bread (TSD-B1) was prepared using 271 g of *Tritordeum* flour, 104 g of tap water, and 125 g of *Tritordeum* sourdough (TSD; 25 % [w/w] of total dough). The second bread (TSD-B2) contained 246 g of *Tritordeum* flour, 104 g of tap water, 125 g of *Tritordeum* sourdough (TSD; 25 % [w/w] of the total dough), and 25 g (5 %, w/w) of *Tritordeum* bran. The third bread (BSD-B3) was prepared using 271 g of *Tritordeum* flour, 104 g of tap water, and 125 g of a *Tritordeum* flour-bran composite sourdough (BSD; 25 % [w/w] of the total dough). The control baker's yeast bread (BYB) was prepared using 333 g of *Tritordeum* flour, 167 g of tap water, and 10 g (2 %) of baker's yeast. The dough was made by mixing the ingredients in a continuous high-speed mixer at $60 \times g$ for 5 min. Fermentation was conducted at 30 ± 1.0 °C for 6 h for sourdoughs and 1.5 h for baker's yeast bread doughs. All breads were baked at 230 °C for 35 min using an Omega 2 oven (Bongard, Italy).

2.5. Textural and nutritional characterization of sourdough breads

Instrumental texture profile analysis (TPA) was done using a TVT 6700 texture analyzer (PerkinElmer) equipped with a 25 mm cylindrical probe (P-CY25S). The test mode settings included a test speed of 1 mm/s, a compression distance of 20 %, and a two-compression cycle (TVT method 01–03.01). TPA data were analyzed using TexCalc 5 software, which measured specific volume, height, width, depth, and area of the loaves. The color was measured using a CR-400 (Konica Minolta, Japan). Samples were placed in petri dishes and filled to the top. The L^* , a^* , and b^* color space analysis method was used, where L^* represents lightness (white, black) and a^* and b^* corresponds to the chromaticity coordinates (red–green and yellow, blue, respectively). Individual free amino acids (FAA) were quantified using the WSEs of freeze-dried bread samples, by the Biochrom 30+ series Amino Acid Analyzer (Biochrom Ltd., UK) equipped with a Li-cation-exchange column (Stringari et al., 2024). The in vitro protein digestibility (IVPD) was determined following the method of Akeson and Stahmann (1964), with modifications as described by Rizzello et al. (2014). The IVPD % was expressed as the ratio of the digested protein fraction to the total protein fraction (Eq. (2))

$$\text{IVPD} = \frac{\text{digested protein}}{\text{total protein}} \times 100 \quad (2)$$

To determine predictive glycemic index (*pGI*) bread samples, containing 1 g of starch were subjected to enzymatic treatment and the released glucose content was measured with α -D-Fructose/D-Glucose Assay Kit (Megazyme). The degree of starch digestion was expressed as the percentage of potentially available starch hydrolyzed after 180 min. Predictive glycemic index (*pGI*) of *Tritordeum* sourdough and baker's yeast bread samples was determined by quantifying the starch hydrolysis index (HI) according to Capriles and Areas (2013) using Eq. (3) as previously proposed Goñi et al. (1997).

$$pGI = 39.71 + 0.549(\text{HI}) \quad (3)$$

The values of pH and TTA, TDF, SDF, ISF, β -glucan, arabinoxylan, carbohydrates, organic acids, ANF, free phenols, total phenols, and DPPH radical scavenging activity were determined as reported above.

2.6. In vitro static digestion simulation

TSD-B1, BSD-B3, and BYB breads were digested following the standardized COST INFOGEST 2.0 protocol (Mackie and Rigby, 2015) with minor modifications (Koirala et al., 2022). Lyophilized breads were mixed 1:2 (w/v) with simulated salivary fluid (SSF, pH 7; KCl 15.10 mM (CAS 7447–40–7), KH_2PO_4 3.70 mM (CAS 7778–77–0, ≥ 99.0 %), NaHCO_3 13.60 mM (CAS 144–55–8, ≥ 99.7 %), $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$ 0.15 mM (CAS 16,674–78–5, ≥ 99.0 %), $(\text{NH}_4)_2\text{CO}_3$ 0.06 mM (CAS 506–87–6, ≥ 99.0 %); supplemented with 0.75 mL α -amylase (1500 U/mL, CAS 9000–90–2) and 41.98 μL CaCl_2 (0.30 M, CAS 10,035–04–8, ≥ 99 %),

and incubated at 37 ± 0.5 °C for 2 min. Gastric digestion was initiated by adding 82.02 mL gastric juice (KCl 0.04 M, NaCl 0.24 M; and 7647–14–5, ≥ 99 %), 6.07 mL pepsin (from porcine gastric mucosa, ≥ 250 U/mg protein, CAS 9001–75–6), and 0.68 mL lecithin (CAS 8002–43–5, ≥ 99 %), followed by 2 h incubation at 37 ± 1.00 °C with pH decreasing from 5.5 to 2.0. For the intestinal phase, 140 mL chyme was combined with 50 mL pancreatic juice (NaHCO_3 14 g/L, CAS 144–55–8; pancreatin 10 g/L, CAS 8049–47–6; Oxgall 15 g/L), 4.50 mL trypsin (from porcine pancreas, 5000 USP U/mg, CAS 9002–07–7), 5.60 mL chymotrypsin (≥ 1000 USP U/mg, CAS 9004–07–3), 180 mg lipase (type II, 100–500 U/mg, CAS 9001–62–1), 300 mg hog α -amylase (50 U/mg, CAS 9000–90–2, Sigma-Aldrich), and 350 μL CaCl_2 (0.30 M). The final 200 mL mixture was incubated for 3 h at 37 ± 1.5 °C under static dialysis (14 kDa MWCO membrane, Spectra/Por[®], Sigma-Aldrich) against 400 mL NaHCO_3 0.04 M (pH 7). The resulting digestates were used as inputs for the SHIME[®] model. All reagents were purchased from Sigma-Aldrich, Steinheim, Germany.

2.7. SHIME[®] simulation

The SHIME[®] (ProDigest, Ghent, Belgium) set up comprised a double-jacketed bioreactor, simulating a stomach and small intestine (ST/SI, pH 2, 37 °C and anaerobic conditions) compartment, that fed three distinct proximal colon (PC) bioreactors (Marzorati et al., 2017). PC bioreactors (500 mL static volume maintained at pH 5.75–5.95, 37 °C and anaerobic conditions) were filled with adult *L*-SHIME[®] medium (PDNM001B) and inoculated with 5 % (v/v) of the same fecal slurry from a previously selected and characterized healthy adult donor (Da Ros et al., 2021) followed by 12 h static incubation for microbiota adaptation. The three PC reactors represented technical replicates fed by the same ST/SI compartment. Biological replication (i.e., independent donors or duplicated SHIME[®] lines) was not feasible due to instrumentation constraints, and this approach aligns with standard SHIME[®] practice in single-donor mechanistic studies (Van de Wiele et al., 2015). A stabilization phase (2 weeks) followed to allow the evolution of a stable colonic community. Then, a control phase (2 weeks) took place during which, short chain fatty acids (SCFA) concentrations, specifically acetic, propionic, and butyric acids, were measured in lumen samples three times weekly to assure reproducibility and stability in PC ecosystems (>90 %) across six consecutive time points. During stabilization and control phases, the ST/SI reactor received 420 mL *L*-SHIME[®] medium (PDNM001B) and 180 mL pancreatic juice (PJ, 12.5 g/L (NaHCO_3 , CAS 144–55–8 ≥ 99.7 %, Sigma-Aldrich, Steinheim, Germany), 6 g/L bile extract (from bovine/porcine bile, Sigma-Aldrich, Steinheim, Germany), 0.9 g/L pancreatin (CAS 8049–47–6, Sigma-Aldrich, Steinheim, Germany) three times daily (every 8 h), retained 45 min before being evenly distributed among the three PC reactors. Then, a treatment phase was performed (7 days) in which 33.3 g per feeding cycle (100 g/day) of each selected pre-digested bread were directly pumped to a different PC bioreactor, followed by a 1-week washout phase in which the same conditions adopted in stabilization and control phases were reinstated. Lumen samples (50 mL) corresponding to the liquid phase contained within each PC bioreactor were collected at the end of control phase (T0; baseline), during treatment (T1, after 3 days; T2, after 7 days), and after 7 days of washout (W1), and stored at -20 °C.

2.8. Short chain fatty acids profile and lactate

Lumen samples collected from each PC bioreactor were centrifuged (Eppendorf 5810R, Germany) at $14,700 \times g$ for 5 min, supernatants filtered and diluted 1:5 in 5 mM H_2SO_4 (≥ 98 %, CAS,7664–93–9, Sigma-Aldrich, Steinheim, Germany). Samples were analyzed on an Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and an UltiMate™ Diode Array Detector. The procedure followed published protocols (Bondue et al., 2020; Tlais et al., 2024) and data were processed

with Chromeleon™ software (Thermo Fisher Scientific, San Jose, CA, USA). Standards of lactic, acetic, propionic, and butyric acids were obtained from Sigma-Aldrich, Steinheim, Germany.

2.9. DNA extraction and 16S rRNA gene sequencing

Lumen samples (4 mL) were centrifuged (14,500 × g, 10 min, RT), and DNA was extracted with the QIAamp PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany) in triplicate. DNA purity and concentration were assessed using Nanodrop One/One (Thermo Fisher Scientific, USA) and Qubit 2.0 (Invitrogen, Italy). DNA was diluted to 5 ng/μL in 10 mM Tris (pH 8.5) (≥ 99.8 %, CAS 77–86–1, Sigma-Aldrich, Steinheim, Germany). The V3–V4 regions of the 16S rRNA gene were amplified using primers. Forward (5′-TCGTCGGCAGCGTCAGATGTGTATAAGA-GACAGCCTACGGGNGGCWGCAG) Reverse (5′-GTCTCGTGGGCTCGGA GATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC). PCR products were purified with AMPure XP beads (Illumina), indexed with Nextera XT, purified again, quantified with Qubit, diluted to 4 nM, pooled (5 μL/sample), spiked with PhiX, and sequenced on an Illumina NextSeq™ 1000 platform (Illumina, San Diego, CA, USA).

2.10. Bioinformatics analysis

Raw reads were processed in R (v4.4.2) using the DADA2 (v1.8) pipeline (Callahan et al., 2016). Reads were filtered and trimmed (truncLen 300/280 bp; trimLeft 8/9 bp), error rates estimated, de-duplicated, denoised, and merged. Chimeras were removed, and ASVs were classified with a naive Bayes classifier trained on SILVA v132 (99 % similarity, V3–V4 region). ASVs present in all replicates at ≥ 0.01 % relative abundance were retained, and samples were rarefied to 205,374 reads. Beta diversity was calculated with Bray-Curtis dissimilarity, visualized by PCoA, and tested with PERMANOVA (adonis2, 999 permutations, Benjamini–Hochberg correction); group dispersion was checked with betadisper. Differential abundance was assessed with MaAsLin2 (v1.8.0), using TSS-normalized, log-transformed genus-level data with treatment and time as fixed effects (BYB and T0 as references). Features with $q < 0.001$ were considered significant. Associations between genera and SCFA concentrations were tested with Spearman correlation ($P < 0.05$).

2.11. Statistical analysis

Fermentations were carried out in triplicate, and each analysis was repeated twice. Data were subjected to one-way and two-way ANOVA; pair-comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$, using the software R version 4.2.2 (R Development Core Team). Principal component analysis (PCA) was performed to explore multivariate patterns of bread samples and Pearson correlation coefficients were computed between DPPH radical-scavenging activity (mM BHT/100 g) and the concentrations of individual free phenolic compounds (mg/kg DW) using pairwise complete observations, and statistical significance was evaluated by two-tailed tests ($P < 0.05$). Both analyses were carried out in OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Microbial, physico-chemical and biochemical characterization of sourdoughs

Compared to the doughs (T0), the cell density of LAB increased by ca. 2 Log CFU/g in mature sourdoughs (T8), regardless of the type of sourdough preparation (TSD and BSD) (Table 1). Similarly, yeast cell numbers increased from ca. 5 Log CFU /g to 7.14 ± 0.11 for TSD and 7.25 ± 0.16 Log CFU /g for BSD. The pH value of doughs prior fermentation was 6.18 ± 0.03 and 5.95 ± 0.02 for TSD and BSD

Table 1

Microbial, physico-chemical, biochemical, antinutritional factors and nutritional characterization of doughs prior fermentation (time = 0 h (T0) and mature sourdoughs (time = 8 h (T8)) prepared with 100 % *Tritordeum* flour (TSD) or *Tritordeum*-bran (1:1) composite (BSD), fermented with *Lactiplantibacillus plantarum* L108.5, *Pediococcus pentosaceus* TLD10–13, *Lactobacillus curvatus* TLD10–17, and *Saccharomyces cerevisiae* TYD10–5. The data are the means of three independent experiments ± standard deviation ($n = 3$). Statistical analysis was performed by one-way ANOVA. Values in the same row with different superscript letters differ significantly ($P < 0.05$).

	TSD		BSD	
	T0	T8	T0	T8
Microbial Characterization				
LAB cell density (Log CFU/g)	7.19 ± 0.07^b	9.13 ± 0.16^a	7.37 ± 0.16^b	9.23 ± 0.17^a
Yeast cell density (Log CFU/g)	5.20 ± 0.08^b	7.14 ± 0.11^a	5.3 ± 0.50^b	7.25 ± 0.60^a
Physico-chemical and biochemical characterization				
pH	6.18 ± 0.03^a	4.21 ± 0.01^c	5.95 ± 0.02^b	4.03 ± 0.01^d
TTA (mL 0.1 M NaOH/10 g)	1.85 ± 0.05^c	11.0 ± 0.50^b	2.55 ± 0.15^c	18.25 ± 0.65^a
Glucose (mM)	14.58 ± 0.56^b	7.63 ± 0.90^c	29.44 ± 0.95^a	14.97 ± 0.70^b
Fructose (mM)	7.15 ± 0.06^c	2.90 ± 0.24^d	31.18 ± 0.84^a	15.59 ± 0.75^b
Maltose (mM)	26.52 ± 1.48^a	5.7 ± 0.25^c	16.04 ± 0.67^b	9.83 ± 0.38^c
Lactic acid (mM)	n.d.	111.99 ± 1.37^b	n.d.	129.26 ± 3.17^a
Acetic acid (mM)	n.d.	21.59 ± 0.49^b	n.d.	23.84 ± 0.53^a
Ethanol (mM)	n.d.	95.22 ± 4.43^a	n.d.	69.9 ± 0.13^b
Fermentation quotient	n.d.	5.19 ± 0.05^b	n.d.	5.42 ± 0.01^a
Antinutritional factors				
Phytic acid (mg/100 g)	397.01 ± 5.29^b	189.98 ± 8.82^c	792.26 ± 22.94^a	290.3 ± 12.94^b
Raffinose oligosaccharide series (mM/100 g)	0.86 ± 0.08^b	0.44 ± 0.01^c	1.64 ± 0.08^a	0.82 ± 0.03^b
Phenolic and Antioxidant characteristics				
Total phenols (mg GAE/kg)	609.0 ± 28.00^c	1032.5 ± 33.50^b	661.5 ± 30.00^c	1445.5 ± 37.50^a
Radical Scavenging activity (DPPH mM BHT/100 g)	5.55 ± 0.03^c	8.55 ± 0.11^b	6.08 ± 0.58^c	10.53 ± 0.32^a
Nutritional characteristics				
Total dietary fiber (%)	2.40 ± 0.01^c	2.45 ± 0.01^c	12.15 ± 0.12^b	13.35 ± 0.10^a
Soluble fiber (%)	0.45 ± 0.05^d	$0.65 \pm 0.01^b^c$	0.75 ± 0.02^b	0.98 ± 0.02^a
Insoluble fiber (%)	1.95 ± 0.05^b	1.80 ± 0.05^c	11.40 ± 0.25^b	12.40 ± 0.10^a
β- glucan (%)	0.38 ± 0.00^b	0.37 ± 0.00^b	0.58 ± 0.01^a	0.60 ± 0.01^a
Arabinoxylin (%)	1.93 ± 0.05^c	2.96 ± 0.13^b	2.50 ± 0.18^c	3.98 ± 0.03^a

respectively, while the pH values decreased in mature sourdoughs, with significant ($P < 0.05$) differences between TSD (4.21 ± 0.01) and BSD (4.03 ± 0.01), while TTA increased correspondingly, with BSD showing the highest value (18.25 ± 0.65 mL 0.1 M NaOH/10 g). Before fermentation, glucose, fructose, and maltose were the predominant carbohydrates, with BSD showing higher initial concentrations of glucose and fructose and TSD having more maltose (Table 1). These sugars were significantly ($P < 0.05$) depleted in mature sourdoughs. Lactic and acetic acids were the primary microbial metabolites, with BSD showing significantly higher ($P < 0.05$) lactic acid than TSD. On the contrary, ethanol concentrations were significantly higher ($P < 0.05$) in TSD compared to BSD. The FQ, which is the ratio of molar concentrations of lactic to acetic acid, also differed significantly between mature

sourdoughs (Table 1).

3.2. Nutritional characteristics and antinutritional factors of sourdoughs

Although with different magnitudes, changes of ANF were found during the fermentation (Table 1). The fermentation had lowered the concentration of phytic acid in both mature sourdoughs (T8), with an overall decrease of 52.1 % in TSD and 63.4 % in BSD as compared to doughs prior to fermentation (T0). In addition to phytic acids, RFO content decreased significantly ($P < 0.05$) during fermentation from 0.86 ± 0.08 to 0.44 ± 0.01 mM/100 g in TSD and 1.64 ± 0.08 to 0.82 ± 0.03 mM/100 g in BSD.

The total phenolic content of TSD and BSD at T0 was 309.0 ± 28 and 560.5 ± 30.5 mg GAE/kg DW respectively, which significantly ($P < 0.05$) increased in mature sourdoughs with the highest value in BSD 1445.5 ± 37.55 mg GAE /kg DW (Table 1). Prior to fermentation (T0), the DPPH radical scavenging activity of TSD was 5.55 ± 0.03 mM BHT/100 g, which significantly increased ($P < 0.05$) to 8.55 ± 0.11 mM BHT/100 g after fermentation (T8). Similarly, BSD exhibited a substantial increase in DPPH radical scavenging activity after fermentation, reaching 10.53 ± 0.32 mM BHT/100 g, which was significantly higher than that of TSD.

Bran addition and sourdough fermentation showed a significant effect on TDF content. Doughs prior fermentation (T0) showed content of TDF 2.40 ± 0.01 for TSD and 12.15 ± 0.25 % DW for BSD, consisting of IDF (1.95 ± 0.05 and 11.35 ± 0.25 % DW) and SDF (0.45 ± 0.05 and 0.80 ± 0.00 % DW) respectively. Overall, BSD had significant ($P < 0.05$) higher amount of TDF, SDF and IDF as compared to TSD. The total β -glucan content of BSD was higher than that of TSD both prior to and after fermentation (Table 1). However, fermentation did not result in significant ($P > 0.05$) changes in β -glucan content for either of the sourdough prototypes. In contrast, the AX content was significantly increased, following fermentation in both TSD and BSD. As expected, BSD exhibited a significantly higher AX content (3.98 ± 0.03 % DW) compared to TSD (2.96 ± 0.13 % DW).

Free phenolic compounds identified and quantified in prototypes prior fermentation and mature sourdoughs are described in Table S1. Protocatechuic acid was identified as a major phenolic compound, showing a significant increase ($P < 0.05$) in mature sourdoughs (T8), with the highest concentration observed in BSD (16.08 ± 1.46 mg/kg DW) followed by TSD (12.01 ± 1.53 mg/ kg DW). Similarly, catechol, procyanidin, vanillic acid, and sinapic acid also increased significantly ($P < 0.05$) in both mature sourdough prototypes. Conversely, ferulic acid and *p*-coumaric acid showed a significant ($P < 0.05$) decrease after fermentation in both mature sourdoughs, with ferulic acid reducing from 7.07 ± 0.51 to 3.85 ± 0.60 mg/ kg DW in TSD and from 10.17 ± 0.75 to 5.68 ± 0.26 mg/ kg DW in BSD, while *p*-coumaric acid decreased from 0.85 ± 0.10 to 0.28 ± 0.00 mg/ kg DW in TSD and from 0.99 ± 0.13 to 0.15 ± 0.01 mg/ kg DW in BSD, respectively. On the other hand, caffeic acid and vanillin showed no significant ($P > 0.05$) difference among all samples regardless of sourdough prototype and time point.

3.3. Physico-chemical, biochemical, and nutritional characterization, of tritordeum sourdough breads

Control bread prepared with *Tritordeum* flour and commercial baker's yeast (BYB) showed the highest pH (5.2 ± 0.10) compared to the sourdough-based breads (Table 2). Among the sourdough breads, the bran-based (BSD-B3; prepared with 25 % BSD) had the lowest pH (4.60 ± 0.11). No significant ($P > 0.05$) differences in pH values were observed between *Tritordeum* sourdough bread (TSD-B1; prepared with 25 % of TSD) (4.61 ± 0.01) and bread prepared with 25 % of TSD and 5 % bran addition (TSD-B2) (4.57 ± 0.02). TTA mirrored the pH values (Table 2). The concentration of lactic acid followed a similar trend, with BSD-B3 presenting the highest value of 28.22 ± 0.9 mM that was significantly ($P < 0.05$) higher than those in TSD-B1 (23.25 ± 0.04 mM)

Table 2

Physico-chemical, biochemical and nutritional characteristics of breads containing 25 % (w/w) *Tritordeum* flour or *Tritordeum* flour-bran composite doughs, fermented with *Lactiplantibacillus plantarum* L108.5, *Pediococcus pentosaceus* TLD10–13, and *Lactilactobacillus curvatus* TLD10–17 along with the yeast strain *Saccharomyces cerevisiae* TYD10–5. TSD1-B1, bread containing 25 % sourdough made with *Tritordeum* flour; TSD-B2, bread containing 25 % sourdough made with *Tritordeum* flour and 5 % bran; BSD-B3, bread containing 25 % sourdough made with *Tritordeum* flour and bran with 1:1 ratio; BYB, control bread made with *Tritordeum* flour and baker's yeast (2 %). The data are the means of three independent experiments \pm standard deviations ($n = 3$). Statistical analysis was performed by one-way ANOVA. Values in the same row with different superscript letters differ significantly ($P < 0.05$).

	TSD-B1	TSD-B2	BSD-B3	BYB
pH	4.81 \pm 0.05 ^c	4.72 \pm 0.10 ^c	4.60 \pm 0.11 ^b	5.20 \pm 0.10 ^a
TTA* (mL 0.1 M NaOH/10 g)	8.3 \pm 0.10 ^b	10.55 \pm 0.45 ^b	11.5 \pm 0.50 ^a	8.50 \pm 0.50 ^b
Lactic acid (mM)	23.2 \pm 0.04 ^b	24.8 \pm 0.16 ^b	28.2 \pm 0.90 ^a	n.d.
Acetic acid (mM)	3.34 \pm 0.05 ^b	3.41 \pm 0.01 ^b	3.52 \pm 0.05 ^a	n.d.
Total phenols (mg GAE /kg)	858 \pm 34.00 ^c	1000.5 \pm 7.50 ^b	1185 \pm 8.00 ^a	631 \pm 4.00 ^d
Radical scavenging activity (DPPH mM BHT/100 g)	7.97 \pm 0.04 ^c	9.23 \pm 0.11 ^b	10.83 \pm 0.13 ^a	6.3 \pm 0.34 ^d
Total dietary fiber (%)	2.95 \pm 0.01 ^d	3.55 \pm 0.05 ^b	4.45 \pm 0.05 ^a	2.80 \pm 0.01 ^c
Soluble fiber (%)	0.50 \pm 0.03 ^c	0.60 \pm 0.02 ^b	0.75 \pm 0.01 ^a	0.40 \pm 0.01 ^d
Insoluble fiber (%)	2.45 \pm 0.05 ^c	2.95 \pm 0.05 ^b	3.70 \pm 0.10 ^a	2.40 \pm 0.05 ^c
β -glucan (%)	0.27 \pm 0.01 ^c	0.33 \pm 0.01 ^b	0.39 \pm 0.01 ^a	0.22 \pm 0.02 ^d
Arabinoxylan (%)	2.75 \pm 0.10 ^b	2.97 \pm 0.21 ^b	3.22 \pm 0.03 ^a	1.99 \pm 0.03 ^c
Phytic acid (mg/100 g)	156.5 \pm 2.29 ^c	186.2 \pm 2.71 ^b	160.2 \pm 4.79 ^c	245.5 \pm 2.03 ^a
Raffinose oligosaccharide series (mM/100 g)	0.37 \pm 0.01 ^c	0.4 \pm 0.01 ^b	0.38 \pm 0.01 ^{bc}	0.50 \pm 0.01 ^a

n.d: not detected.

TTA: Total titratable acidity.

and TSD-B2 (24.84 ± 0.16 mM). Conversely, no significant difference in lactic acid was observed between TSD-B1 and TSD-B2. Likewise, acetic acid was also significantly ($P < 0.05$) higher in BSD-B3 (3.52 ± 0.01 mM) than in TSD-B1 (3.34 ± 0.03 mM) and TSD-B2 (3.41 ± 0.01 mM). Organic acids were not found in BYB (Table 2).

The free phenolic compounds concentrations trends, except for vanillin that was not detected in TSD-B1 and BYB bread samples, were like those previously reported in mature sourdoughs (Table S2). In general BSD-B3 bread samples showed the highest concentration of free phenolic compounds followed by TSD-B2 and TSD-B1, while the lowest content was detected in BYB. Vanillic acid was the predominant compound with a highest value of 4.40 ± 0.16 mg/kg in BSD-B3. Caffeic acid and procyanidin-B3 followed the same trend. BSD-B3 also showed the significantly higher values for ferulic and sinapic acids (3.94 ± 0.14 , 1.24 ± 0.10 mg/kg DW, respectively), while there were no significant ($P > 0.05$) differences between TSD-B1 and TSD-B2. Interestingly, vanillin was only detected in bran-based samples with the value of 0.60 ± 0.05 and 0.39 ± 0.03 mg/kg DW in BSD-B3 and TSD-B2, respectively. Furthermore, the total phenolic content followed the same trend observed in the phenolic profile. Compared with BYB, all sourdough breads presented significantly higher total phenolic content ($P < 0.05$). Among them, BSD-B3 showed the highest value (1185 ± 8.5 mg GAE/kg), followed by TSD-B2 (1000.5 ± 7.5 mg GAE/kg) and TSD-B1 (858 ± 34.5 mg GAE/kg). A similar trend was observed in DPPH radical scavenging activity, where BSD-B3 showed the highest value 10.83 ± 0.13 mM BHT/100 g significantly ($P < 0.05$) surpassing TSD-B2 (9.23 ± 0.11 mM BHT /100 g), and TSD-B1 (7.97 ± 0.04 mM BHT/100 g), while the

lowest value was observed in BYB 6.3 ± 0.34 mM BHT/100 g (Table 2). Moreover, BYB had the highest phytic acid content (245.53 ± 2.03 mg/100 g), whereas the lowest value was found in TSD-B1 (156.51 ± 2.29 mg/100 g) (Table 2). No significant difference was found between TSD-B1 and BSD-B3 in terms of phytic acid content. However, TSD-B2 had a significantly higher phytic acid concentration (186.22 ± 2.71 mg/100 g) compared to both TSD-B1 and BSD-B3. RFO content varied from 0.37 ± 0.01 to 0.50 ± 0.0 mM/100 g among the sourdough-based breads, with no significant differences ($P > 0.05$). However, a significant difference ($P < 0.05$) was noted between BYB and sourdough-based breads (Table 2). Correlation analysis showed that the antioxidant capacity of *Tritordeum* breads, measured by DPPH radical-scavenging activity, was strongly associated with specific free phenolic compounds (Figure S1). Significant positive correlations ($p < 0.05$) were observed for p-coumaric acid ($r = 0.999$), procyanidin-B3 ($r = 0.987$), vanillic acid ($r = 0.964$), caffeic acid ($r = 0.953$), and ferulic acid ($r = 0.949$). Other phenolics showed positive but non-significant relationships.

3.4. Non starch polysaccharides

TDF results highlight statistically significant differences ($P < 0.05$) between sourdough breads and *Tritordeum* baker's yeast bread. BSD-B3 had the highest TDF content (4.45 ± 0.05 % DW), followed by TSD-B2 (3.55 ± 0.05 % DW), TSD-B1 (2.95 ± 0.01 % DW), and the lowest in BYB (2.80 ± 0.01 % DW) (Table 2). The same significant ($P < 0.05$) differences were found for SDF. Similarly, BSD-B3 showed the highest IDF value (3.7 ± 0.1 % DW), significantly greater ($P < 0.05$) than all other bread types, with BYB having the lowest value (2.60 ± 0.05 % DW). The β -glucan content of the breads ranged from 0.39 to 0.22 % DW, where the highest content was found in BSD-B3 (0.39 ± 0.01 %), and the lowest value in BYB (0.22 ± 0.00 % DW). All breads had significantly different content of β -glucan among them. Likewise, AX content was highest in BSD-B3 (3.22 ± 0.03 % DW), followed by TSD-B2 (2.97 ± 0.01 % DW), TSD-B1 (2.75 ± 0.10 %), and the lowest in BYB (1.99 ± 0.03 %). Notably, the AX content in TSD-B2 and TSD-B1 showed no significant difference ($P > 0.05$), whereas variations were observed among the other breads.

3.5. Amino acid profiling, in vitro protein digestibility and predictive glycemic index (IVPD & pGI)

As expected, sourdough breads had a higher FAA concentration compared to the control bread (Table 3). Moreover, BSD-B3 had the highest value ($P < 0.05$) of total FAA (1758.02 ± 6.56 mg/kg DW) followed by TSD-B2 (1670.94 ± 6.88 mg/kg DW), and TSD-B1 (1325.17 ± 15.64 mg/kg DW). Gly, Ala, Ile, Leu, Phe, and Lys increased in the bread samples with bran as compared to TSD-B1 and baker's yeast bread (BYB). Similarly, an upward trend was observed for Val, Ile, Leu and GABA in sourdough bread. Moreover, BSD-B3 had the highest levels of several essential amino acids such as Thr, Val, Leu, Phe, Lys, and His and significantly ($P < 0.05$) differing from the other sourdough and baker's yeast bread.

The IVPD of breads were markedly affected by the addition of bran and sourdough fermentation (Table 3). Among all breads TSD-B1, showed significant ($P < 0.05$) the highest value of IVPD (78.76 ± 0.12 %) followed by TSD-B2 (77.2 ± 0.21 %), BSD-B3 (75.34 ± 0.16 %) and BYB (64.02 ± 0.40 %). The fermented bran-based sourdough had the most noticeable effect in pGI, with BSD-B3 showing the lowest value (62.76 ± 0.58 %), followed by TSD-B2 (65.39 ± 0.17 %) and TSD-B1 (67.35 ± 0.29 %). The highest pGI was observed in BYB (71.59 ± 0.39 %).

3.6. Texture profile and color analysis of breads

Compared to BYB (2.42 ± 0.05 cm³/g), the specific volume of sourdough-based breads was slightly but significantly ($P < 0.05$) lower

Table 3

Concentrations of individual free amino acids (FAA), total free amino acids (TFAA), and their derivatives (mg/kg), together with *in vitro* protein digestibility (IVPD, %, dry weight) and predicted glycaemic index (pGI, %, dry weight), for breads containing 25 % (w/w) *Tritordeum* sourdough fermented with *Lactiplantibacillus plantarum* LM108.5, *Pediococcus pentosaceus* TLD10–13, *Lactilactobacillus curvatus* TLD10–17, and *Saccharomyces cerevisiae* TYD10–5. Bread codes: TSD-B1, *Tritordeum* bread containing 25 % sourdough made with *Tritordeum* flour; TSD-B2, *Tritordeum* bread containing 25 % sourdough and 5 % (w/w) *Tritordeum* bran; BSD-B3, *Tritordeum* bread containing 25 % sourdough made from a *Tritordeum* flour–bran composite (1:1, w/w); BYB, control bread made with *Tritordeum* flour and 2 % (w/w) baker's yeast. Data are presented as means \pm standard deviations ($n = 3$). Statistical analysis was performed using one-way ANOVA. Values within the same row followed by different superscript letters differ significantly ($p < 0.05$).

FAA (mg/kg)	TSD-B1	TSD-B2	BSD-B3	BYB
Asp	90.59 \pm 2.61 ^b	86.21 \pm 0.02 ^{bc}	129.47 \pm 0.09 ^a	82.99 \pm 0.19 ^c
Thr	77.26 \pm 2.85 ^a	55.12 \pm 0.74 ^b	86.01 \pm 1.48 ^a	71.88 \pm 5.24 ^{ab}
Ser	23.25 \pm 0.04 ^b	29.99 \pm 0.84 ^a	28.22 \pm 0.9 ^a	3.10 \pm 0.1 ^c
Asn	3.74 \pm 0.02 ^a	3.52 \pm 0.01 ^b	3.52 \pm 0.01 ^b	N. d
Glu	229.72 \pm 1.71 ^a	212.98 \pm 0.75 ^b	216.21 \pm 5.88 ^b	142.23 \pm 1.72 ^c
Gly	36.5 \pm 0.82 ^b	36.6 \pm 0.65 ^b	64.44 \pm 0.93 ^a	35.08 \pm 0.27 ^b
Ala	113.76 \pm 0 ^c	180.48 \pm 0.09 ^a	137.66 \pm 1.55 ^b	18.55 \pm 3.34 ^d
Val	53.06 \pm 1.65 ^b	72.54 \pm 2.09 ^a	73.69 \pm 2.93 ^a	33.41 \pm 1.02 ^c
Cys	27.46 \pm 0.06 ^a	30.89 \pm 2.69 ^a	31.02 \pm 3.09 ^a	8.52 \pm 0.52 ^b
Met	46.72 \pm 0.58 ^a	15.67 \pm 4.37 ^b	52.71 \pm 1.72 ^b	5.87 \pm 1.34 ^b
Ile	60.05 \pm 1.48 ^b	99.32 \pm 1.72 ^a	102.09 \pm 1.64 ^a	52.26 \pm 1.00 ^b
Leu	138.5 \pm 2.48 ^c	228.23 \pm 0.72 ^a	172.64 \pm 1.64 ^b	27.42 \pm 3.99 ^d
Tyr	99.67 \pm 3.86 ^b	120.81 \pm 9.38 ^b	167.84 \pm 4.53 ^b	51.56 \pm 1.50 ^c
Phe	20.52 \pm 3.85 ^{ab}	30.11 \pm 5.75 ^a	15.25 \pm 0.31 ^{ab}	8.3 \pm 2.51 ^b
Orn	4.06 \pm 0.17 ^a	9.11 \pm 4.88 ^a	17.17 \pm 0.17 ^a	11.49 \pm 0.23 ^a
Lys	27.61 \pm 2.19 ^b	36.48 \pm 4.28 ^{ab}	49.08 \pm 1.83 ^a	29.33 \pm 3.31 ^b
His	58.37 \pm 1.94 ^b	66.33 \pm 0.98 ^a	51.6 \pm 0.19 ^b	13.12 \pm 0.97 ^c
Arg	66.13 \pm 0.79 ^{ab}	54.27 \pm 4.56 ^b	71.73 \pm 3.31 ^a	20.75 \pm 1.64 ^c
Pro	31.08 \pm 4.56 ^a	39.75 \pm 1.07 ^a	54.19 \pm 0.69 ^a	49.09 \pm 7.30 ^a
GABA	73.95 \pm 0.74 ^c	109.07 \pm 0.86 ^b	145.18 \pm 1.29 ^a	60.19 \pm 0.72 ^d
TFAA	1307.52 \pm 15.64 ^c	1576.08 \pm 6.88 ^b	1758.02 \pm 6.56 ^a	798.21 \pm 7.89 ^d
IVPD (% DW)	78.76 \pm 0.12 ^a	75.34 \pm 0.16 ^c	77.2 \pm 0.21 ^b	64.02 \pm 0.40 ^d
pGI (% DW)	67.35 \pm 0.29 ^b	65.39 \pm 0.17 ^b	62.76 \pm 0.58 ^c	71.59 \pm 0.39 ^a

in, TSD-B1, TSD-B2, and BSD-B3 (2.26 ± 0.05 , 2.08 ± 0.02 , and 1.95 ± 0.05 cm³/g, respectively). No significant ($P > 0.05$) difference was observed between TSD-B1 and BSD-B3 sourdough breads for specific volume (Table 4). BYB showed the highest value for resilience and cohesiveness, while BSD-B3 had the lowest. However, there was no significant difference among sourdough bread was found (Table 4). Regarding springiness, there was no significant difference ($P > 0.05$) observed across all bread samples. Moreover, in bran-based sourdough breads, firmness and gumminess increased respectively when bran or fermented bran-based sourdough was used as ingredient in breadmaking (Table 4). Furthermore, the incorporation of bran or bran-based sourdough affected the color of the crumb, leading to a decrease of lightness (L) and to an increase of the value of b and ΔE (Table 4).

Table 4

Texture characteristics and color analysis of breads containing 25 % (w/w) *Tritordeum* flour or *Tritordeum* flour-bran composite doughs, fermented with *Lactiplantibacillus plantarum* LM108.5, *Pediococcus pentosaceus* TLD10–13, and *Latilactobacillus curvatus* TLD10–17 along with the yeast strain *Saccharomyces cerevisiae* TYD10–5. Bread codes: TSD-B1, *Tritordeum* bread containing 25 % sourdough made with *Tritordeum* flour; TSD-B2, *Tritordeum* bread containing 25 % sourdough and 5 % (w/w) *Tritordeum* bran; BSD-B3, *Tritordeum* bread containing 25 % sourdough made from a *Tritordeum* flour–bran composite (1:1, w/w); BYB, control bread made with *Tritordeum* flour and 2 % (w/w) baker’s yeast. The data are the means of three independent experiments ± standard deviations ($n = 3$). Statistical analysis was performed by one-way ANOVA. Values in the same row with different superscript letters differ significantly ($P < 0.05$).

	TSD-B1	TSD-B2	BSD-B3	BYB
Specific Volume (cm ³ /g)	2.28 ± 0.07 ^b	2.08 ± 0.02 ^c	1.95 ± 0.05 ^c	2.42 ± 0.05 ^a
Resilience	0.71 ± 0.01 ^{ab}	0.62 ± 0.03 ^{bc}	0.62 ± 0.02 ^c	0.73 ± 0.01 ^a
Cohesiveness	0.45 ± 0.05 ^b	0.46 ± 0.04 ^{ab}	0.45 ± 0 ^b	0.65 ± 0.05 ^{ab}
Springiness	1.00 ± 0.01 ^a	0.95 ± 0.05 ^a	0.90 ± 0.05 ^a	1.00 ± 0.01 ^a
Gumminess (g)	853 ± 10.00 ^c	1024 ± 8.0 ^b	1237 ± 3.00 ^a	531 ± 1.00 ^e
Firmness (g)	1786 ± 3.0 ^c	2244 ± 8.0 ^a	2129.5 ± 15.5 ^b	1565 ± 10.00 ^d
Crumb Color				
L*	67.30 ± 0.41 ^a	59.02 ± 0.64 ^b	56.40 ± 0.30 ^c	67.95 ± 0.55 ^a
a*	1.40 ± 0.02 ^c	2.38 ± 0.03 ^b	2.63 ± 0.02 ^a	1.38 ± 0.04 ^c
b*	20.80 ± 0.52 ^c	23.77 ± 0.22 ^a	23.40 ± 0.20 ^{ab}	20.70 ± 0.32 ^c
ΔE	33.50 ± 0.50 ^d	45.00 ± 0.15 ^b	51.35 ± 0.35 ^a	35.75 ± 0.45 ^c

3.7. Principal component analysis (PCA) of bread characteristics

PCA was performed to discern the relationships among the biochemical and textural characteristics of bread made with *Tritordeum* flour and bran composites (Fig. 1) and to further guide the selection of bread formulations for the SHIME[®] experiment. Cumulatively the first two principal components explained 97.46 % of the total variance among the samples. The first principal component (PC1) accounted for 86.55 % of the variance and was strongly correlated with biochemical attributes such as TDF, SDF, IDF, total phenolic content, β-glucans, arabinoxylans, DPPH, FFA, as well as firmness and gumminess. The second principal component (PC2), explaining 10.91 % of the variance, was associated with textural attributes such as cohesiveness, resilience, and specific volume, alongside *pGI*. The PCA plot distinctly positioned BYB away from the sourdough variants, primarily due to its higher raffinose and phytic acid content and weaker content in fiber fractions and phenolics. In contrast, breads containing bran (TSD-B2 and BSD-B3), particularly BSD-B3, clustered closely together on the plot, indicating similar profiles characterized by higher DF and phenolics, illustrating the impact of bran addition and sourdough fermentation on bread properties. TSD-B1 appeared as the most distinct formulation, separated far along negative PC1, reflecting its unique profile with higher specific volume and resilience but lower content in fiber fractions and phenolics. Based on these results, all bread types were selected for subsequent SHIME[®] analysis, with TSD-B1 being retained as a contrasting sample and TSD-B2 being excluded due to its strong similarity to BSD-B3.

3.8. SCFA profiles and lactate during SHIME[®] feeding

SCFA and lactate profiles in the proximal colon showed significant effects of time and treatment, with distinct patterns across compounds (Table 5). Acetate concentration peaked at T1 in all PC bioreactors, with significantly higher levels in TSD-B1 compared to BSD-B3 and BYB ($P < 0.05$). At T2, acetate remained elevated but was highest in BSD-B3 and

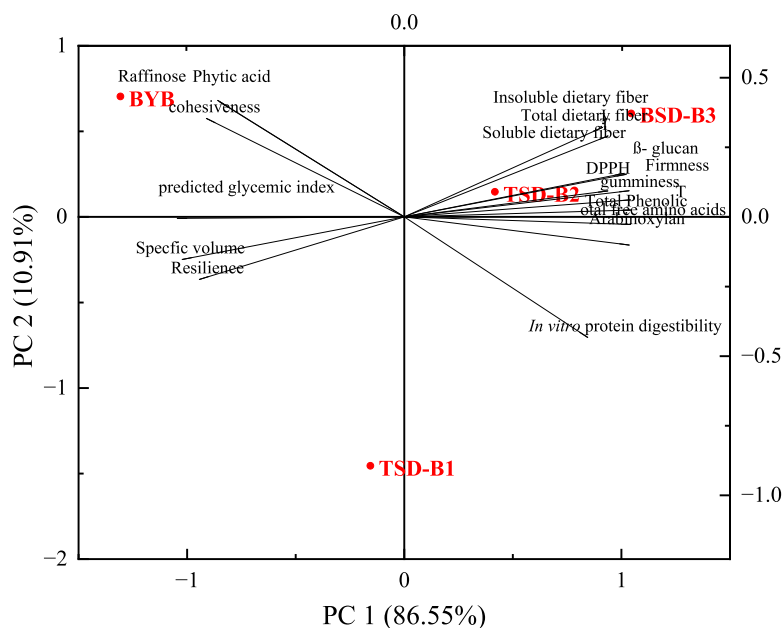


Fig. 1. Principal component analysis (PCA) based on the nutritional variables—total phenolic content (TPC), DPPH radical scavenging activity (DPPH), total dietary fiber (TDF), soluble dietary fiber (SDF), insoluble dietary fiber (IDF), β-glucan, arabinoxylan (AX), phytic acid, raffinose, total free amino acids (TFAA), in vitro protein digestibility (IVPD), predicted glycaemic index (*pGI*)—and textural characteristics including specific volume, resilience, cohesiveness, gumminess, and firmness of *Tritordeum* breads (dough yield, DY 150). All sourdough-based breads contained 25 % (w/w) sourdough fermented with *Lactiplantibacillus plantarum* LM108.5, *Pediococcus pentosaceus* TLD10–13, *Latilactobacillus curvatus* TLD10–17, and *Saccharomyces cerevisiae* TYD10–5. Bread codes: TSD-B1, *Tritordeum* bread with 25 % sourdough; TSD-B2, *Tritordeum* bread with 25 % sourdough plus 5 % (w/w) *Tritordeum* bran; BSD-B3, *Tritordeum* bread with 25 % sourdough prepared from a *Tritordeum* flour–bran composite (1:1, w/w); BYB, baker’s yeast bread prepared with *Tritordeum* flour (2 % yeast, DY 150), used as control.

Table 5

Short chain fatty acid (SCFA) and Short-chain hydroxy acid (mM) in proximal colon (PC) tract of the SHIME model, at different time point before (T0), after three (T1) and seven (T2) days before and end washing out (W1) while fed with digested bread (TSD-B1, BSD-B3, BYB). Bread codes: TSD-B1, *Tritordeum* bread containing 25 % (w/w) sourdough made with *Tritordeum* flour; BSD-B3, *Tritordeum* bread containing 25 % sourdough made from a *Tritordeum* flour-bran composite (1:1, w/w); BYB, control bread made with *Tritordeum* flour and 2 % (w/w) baker's yeast. Values are presented as mean \pm standard deviation. Different lowercase letters within the same column indicate significant differences between treatments at the same time point ($P < 0.05$). Different uppercase letters within the same rows indicate significant differences over time within each treatment ($P < 0.05$). Statistical comparisons were performed using two-way ANOVA.

SCFA (mM)	Treatment times	PC1 (TSD-B1)	PC2 (BSD-B3)	PC3 (BYB)
Acetate	T0	47.40 \pm 0.81 ^{cA}	45.57 \pm 0.69 ^{dA}	47.47 \pm 1.05 ^{cdA}
	T1	344.17 \pm 1.95 ^{aA}	294.42 \pm 0.83 ^{aC}	322.19 \pm 2.94 ^{aB}
	T2	194.51 \pm 0.74 ^{bb}	238.55 \pm 1.69 ^{bA}	178.23 \pm 0.58 ^{bc}
	W1	47.90 \pm 0.86 ^{cB}	47.54 \pm 0.43 ^{cB}	50.55 \pm 0.46 ^{cA}
Propionate	T0	14.44 \pm 0.25 ^{dB}	18.80 \pm 1.22 ^{aA}	15.07 \pm 0.35 ^{cB}
	T1	8.24 \pm 0.32 ^{cB}	12.30 \pm 0.49 ^{aC}	8.40 \pm 0.32 ^{dB}
	T2	112.92 \pm 0.74 ^{aA}	5.55 \pm 0.29 ^{dC}	92.10 \pm 0.50 ^{aB}
	W1	41.58 \pm 0.32 ^{bA}	38.00 \pm 0.54 ^{aB}	41.55 \pm 1.28 ^{bA}
Butyrate	T0	33.92 \pm 1.36 ^{aA}	34.19 \pm 1.93 ^{bA}	33.37 \pm 1.50 ^{aA}
	T1	25.60 \pm 1.22 ^{bb}	41.61 \pm 1.99 ^{aA}	25.81 \pm 1.94 ^{bb}
	T2	25.01 \pm 0.84 ^{bb}	29.45 \pm 0.41 ^{cA}	24.79 \pm 0.45 ^{bb}
	W1	3.96 \pm 1.06 ^{cB}	11.50 \pm 0.83 ^{dA}	5.63 \pm 1.63 ^{cB}
Short-chain hydroxy acid				
Lactate	T0	n.d	n.d	n.d
	T1	189.03 \pm 0.59 ^{aB}	240.0 \pm 1.90 ^{bA}	187.5 \pm 2.05 ^{aB}
	T2	181.65 \pm 0.59 ^{bb}	249.0 \pm 1.90 ^{aA}	184.8 \pm 1.20 ^{aB}
	W1	2.69 \pm 0.21 ^{cB}	1.49 \pm 0.05 ^{cA}	2.33 \pm 0.30 ^{bb}

n.d: not detected.

returned to treatment-specific baseline levels by W1. Propionate exhibited a delayed peak at T2 (with exception BSD-B3 which peaked at W1), where TSD-B1 and BYB reached significantly higher concentrations than BSD-B3. Butyrate levels progressively declined from T0 to W1, with BSD-B3 maintaining significantly higher levels at each time point. Lactate, undetectable at baseline, accumulated markedly at T1 and T2, particularly in BSD-B3, and dropped near baseline by W1.

3.9. Colon microbiome evolution

Fig. 2A shows the twenty most abundant bacterial genera identified in the proximal colon compartment. At baseline (T0), the microbiota was dominated by *Bifidobacterium* (52.0–63.2 %), followed by *Bacteroides* (10.0–16.2 %), *Acidaminococcus* (5.6–10.6 %), *Dialister* (5.3–6.5 %), and *Collinsella* (3.2–3.8 %). *Blautia* was also detected at lower levels (3.3–4.2 %). Following treatment (T1), *Acidaminococcus* increased markedly, peaking at 23.5 % in BSD-B3 and remaining elevated through T2 and W1. *Bifidobacterium* further increased under BYB (79.2 %) and TSD-B1 (77.5 %). *Megasphaera* also increased transiently at T1 under BSD-B3 (8.6 %) and TSD-B1 (7.3 %). In contrast, *Bacteroides* declined below 1 % by T1 and remained low through W1, with only minor

recovery (0.3–0.6 %). *Blautia* followed a similar trend, becoming undetectable by T2. At W1, *Bifidobacterium* remained dominant (54.1–59.9 %), while *Collinsella* and *Acidaminococcus* persisted at moderate levels. Shannon diversity significantly differed by time and treatment (two-way ANOVA, $P < 0.001$). All treatments showed a decrease from T0 to T1 ($P < 0.001$), with a continued decrease in BYB until T2 and partial recovery at W1. TSD-B1 showed an initial drop followed by gradual recovery, while BSD-B3 showed a more moderate but sustained reduction through W1 (Fig. 2B). PCoA based on Bray–Curtis distances showed clear separation by time and partial overlap across treatments (Fig. 2C). Beta diversity confirmed a strong effect of time (PERMANOVA: $F = 18.57$, $R^2 = 0.589$, $p < 0.001$) and no effect of treatment ($F = 1.14$, $R^2 = 0.064$, $p > 0.001$). Pairwise differences were significant between all the time points ($p < 0.001$), while treatment comparisons remained non-significant. Group dispersion was homogeneous (PERMDISP: $F = 0.08$, $p > 0.001$).

MaAsLin2 identified significant associations between specific genera and time ($q < 0.001$), especially at T2 and W1 (Fig. 2D and Supplementary Figure 1). *Bacteroides* decreased most strongly at T2 (coef = -14.18), followed by *Megasphaera*, *X. Eubacterium hallii* group, and *Anaeroglobus* (coef < -10). Other reduced genera included *Selenomonas*, *Centipeda*, *Parasutterella*, *Agathobacter*, and *Blautia* (coef = -9.03 to -7.84). In contrast, *Enterobacter* increased from T1 to W1 (coef = 2.72 to 7.19), as did *Lysinibacillus* and *Weissella*. *Bifidobacterium* increased at T1 and T2 (coef = 0.35 and 0.23) and decreased under BSD-B3 (-0.18). Correlations between genera and metabolites (Fig. 2E) showed positive associations of *Bifidobacterium* with acetate and lactate ($r > 0.78$, $p < 0.01$). *Collinsella* correlated with multiple SCFAs in BSD-B3 ($r > 0.61$). *Enterobacter* correlated positively with propionate and negatively with butyrate ($r > 0.89$, $p < 0.001$). *Weissella* correlated positively with lactate and propionate ($r > 0.70$). A summary visualization integrating SCFA profiles with temporal changes in the genera significantly associated with treatment or time (MaAsLin2, $q < 0.05$) is provided in Figure S2.

4. Discussion

Although cereal-based baked goods rich in DF and bioactive compounds may contribute to reducing chronic disease risk, DF intake remains insufficient in many populations (Stephen et al., 2017). Alternative cereals and their by-products, such as bran, represent opportunities to improve nutritional quality while supporting environmental sustainability (Papadopoulos et al., 2024), with *Tritordeum* proposed as a partial replacement for wheat (Suchowilaska et al., 2021). In addition to exploiting this cereal, our study aimed to valorize its milling by-product, bran, by developing a *Tritordeum* sourdough bread formulation incorporating this ingredient. The effects on gut microbial composition were assessed using the *in vitro* SHIME[®] model.

Fermentation of *Tritordeum* flour and *Tritordeum*-bran blend supported robust LAB growth and acidification, with increases of 2 log CFU/g in cell density, consistent with typical cereal sourdoughs (Arora et al., 2021). Both prototypes (TSD and BSD) underwent marked biochemical changes, but extra bran addition in sourdough (BSD) preparation provided higher initial carbohydrate levels (glucose, maltose, and fructose), resulting in stronger microbial activity. Consequently, BSD showed greater acidification, reflected by lower pH, higher TTA, and elevated lactic and acetic acids, in line with previous reports that bran enhances microbial acid production (İncili et al., 2025; Jing et al., 2024). This may also reflect bran-associated enzymatic activities, such as phytase and feruloyl esterase, which release fermentable substrates. Fermentation quotients were similar (5.1 in TSD, 5.4 in BSD), close to the recommended value of 5.0 for wheat-based sourdough (Arora et al., 2021), and higher than those reported in spontaneous *Tritordeum* sourdoughs (3.5–4.0) after prolonged propagation (Arora et al., 2022). The slightly higher lactic/acetic ratio (FQ) observed in BSD (5.4) indicates a metabolic shift toward homofermentative lactic acid production in the bran-enriched environment. The presence of bran increases the

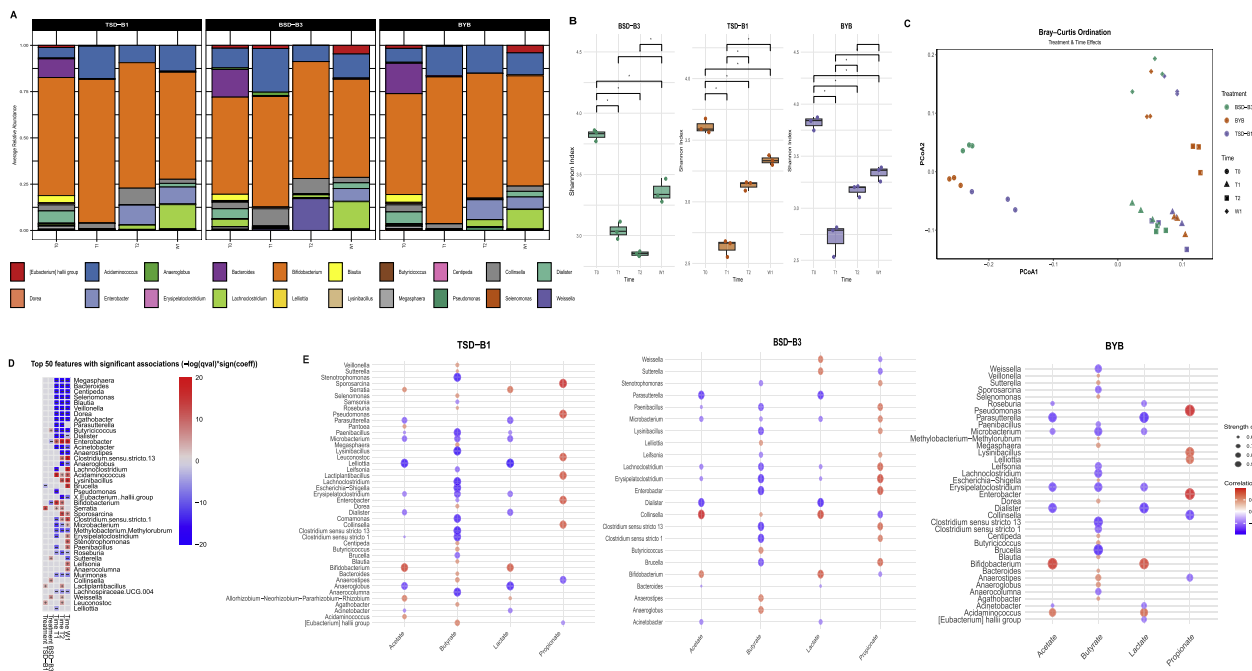


Fig. 2. Microbial community dynamics and metabolite associations in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) following treatment with *Tritordeum* breads. Treatments included TSD-B1, *Tritordeum* sourdough bread prepared with 25 % (w/w) *Tritordeum* sourdough; BSD-B3, *Tritordeum* bran-based sourdough prepared with 25 % (w/w) *Tritordeum* flour–bran composite sourdough (1:1, w/w); and BYB, baker’s yeast bread prepared with *Tritordeum* flour (2 % w/w baker’s yeast, DY 150). Time points included baseline (T0), day 3 (T1) and day 7 (T2) of treatment, and the end of the washout period (W1). (A) Relative abundance of the 20 most dominant bacterial genera across treatments and time points. (B) Shannon diversity index calculated for each treatment over time, with asterisks indicating significant differences (two-way ANOVA, $p < 0.001$). (C) Principal coordinates analysis (PCoA) using Bray–Curtis distances showing separation of microbial communities over time and partial overlap across treatments (PERMANOVA, $F = 18.57$, $R^2 = 0.589$, $p < 0.001$). (D) Heatmap of genera significantly associated with treatment and time identified by MaAsLin2 ($q < 0.001$), showing positive and negative coefficients across bread types and fermentation phases. (E) Correlation matrix displaying associations between major bacterial genera and short-chain fatty acids (acetate, propionate, butyrate) and lactate, with correlation strength indicated by color scale ($r > 0.6$ shown).

buffering capacity of the dough and provides additional fermentable carbohydrates and minerals that sustain glycolytic activity and stabilize intracellular pH. These conditions favor the Embden–Meyerhof and reduce the need for secondary electron-accepting routes that yield acetic acid, thus promoting lactic acid dominance. It reflects the ability of the LAB consortium to adapt its metabolism to the nutrient-rich and buffered bran matrix (De Vuyst et al., 2023). Overall, *Tritordeum*–bran sourdough promoted higher LAB growth and acidification, driven by enhanced sugar availability and metabolic activity, with potential implications for extended shelf-life through inhibition of spoilage agents (Stringari et al., 2024).

Although bran incorporation may enhance the nutritional value of bread, the bioavailability of several nutrients is limited by ANF, particularly phytic acid (Mushtaq et al., 2025). In our study, BSD showed a 63 % reduction in phytic acid, which is in line with Pontonio et al. (2020) who reported that fermenting wheat, barley, and emmer brans with a selected LAB starter (*L. plantarum* T6B10 and *Weissella confusa* BAN8) decreased phytic acid by 24–60 % across brans after 24 h and when these fermented brans were incorporated (30 % w/w) into bread, phytic acid was found lower than in baker’s yeast controls. This is likely due to combined activity of endogenous and LAB-derived phytases, consistent with previous reports (Huang et al., 2024; Kumar et al., 2010). RFO decreased by 48 % in TSD and 50 % in BSD compared to doughs prior fermentation, consistent with previous reports on sourdough wheat doughs (Menezes et al., 2019) with reductions up to 39 % after 4 h and up to 69 % after 12 h of fermentation. This effect can be attributed to LAB α -galactosidase, which hydrolyzes α –1,6 linkages of galactooligosaccharides (Rizzello et al., 2010), with possible additional contributions from fructansucrases (Kahala et al., 2021).

Sourdough fermentation increased total phenolic compounds and antioxidant activity, with higher values observed in both *Tritordeum* and

especially in bran-based sourdoughs. This effect likely reflects the activity of LAB enzymes such as β -glucosidase and feruloyl esterase, which hydrolyze complex or glycosylated phenolics into their free acid forms, thereby enhancing solubility and radical scavenging potential (Li et al., 2024b). Similarly, a stronger antioxidant potential in bran-based sourdoughs was shown (Rizzello et al., 2012), supporting the contribution of bran incorporation to this effect. Targeted profiling revealed nine detectable phenolic compounds, with protocatechuic acid showing the highest concentration, consistent with the naturally high levels previously reported in *Tritordeum* (Montesano et al., 2021).

Fermentation led to a marked increase in protocatechuic acid and catechol, changes that can be mechanistically linked to ferulic acid metabolism by LAB esterases and decarboxylases (Landete et al., 2021). Protocatechuic acid is known for its potent free radical scavenging activity, metal chelation, and ability to enhance endogenous antioxidant enzymes, thereby mitigating oxidative stress (Zhang et al., 2021). Catechol, a downstream metabolite, also contributes to antioxidant activity via efficient radical neutralization. In addition, procyanidin, vanillic acid, and sinapic acid increased after fermentation, with more pronounced effects in BSD. These shifts likely reflect microbial breakdown of proanthocyanidins and enzymatic hydrolysis of glycosylated forms, processes known to enhance polyphenol bioavailability and antioxidant potential (Qin et al., 2025). Vanillic and sinapic acids exhibit significant antioxidant capacities by donating electrons and inhibiting lipid peroxidation, while procyanidins are strong radical scavengers (Boudaoud et al., 2021). Importantly, the compound-specific increases observed in BSD paralleled their higher antioxidant activity, underlining the added value of bran incorporation beyond what is captured by total phenolic content. As expected, BSD contained higher DF concentrations than TSD, pointing to the breakdown of complex polysaccharides into soluble forms during fermentation. This was

evident in the increase of SDF, particularly in BSD, consistent with findings on *Tritordeum* type I sourdough (Arora et al., 2022). In contrast, β -glucan content remained unchanged, in line with oat-based sourdough results (Cera et al., 2024). This outcome differs from wheat and oat bran sourdoughs, where decreases were linked to β -glucan-degrading enzymes that depolymerize the polysaccharide and lower its molecular weight (Gamel et al., 2015). To date, no studies have specifically examined β -glucan dynamics in *Tritordeum* sourdough, but the higher levels observed in BSD likely reflect its greater overall DF. AX content also increased after fermentation, in agreement with rye sourdough studies (Koj and Pejcz, 2023), likely due to xylanase activity within the acidic pH range of sourdough fermentation (Reffai and Fechtali, 2025). These modifications in fiber composition were mirrored in the breads, where bran-containing formulations consistently exhibited improved nutritional profiles compared with baker's yeast bread.

The phenolic profile of bread confirmed the dominance of vanillic and ferulic acids, both of which reached higher concentrations in bran-based sourdough bread (BSD-B3). The enrichment of these compounds is consistent with the presence of vanillic acid in cereal bran and the release of bound ferulic acid during fermentation, processes that are further stabilized by their relative thermal resistance (Menga et al., 2010; Zhang et al., 2023). The detection of vanillin only in bran-containing breads suggests a strong association with bran matrices or a higher susceptibility to degradation during baking in formulations without bran. Beyond these compounds, BSD-B3 also exhibited the greatest increases in protocatechuic acid, caffeic acid, and procyanidin-B3, indicating that bran addition and fermentation act synergistically to enhance both the diversity and availability of phenolics. These findings reinforce previous evidence that sourdough fermentation promotes the enzymatic release of soluble and bound phenolics (Ganzle, 2014).

To further support these findings, Pearson correlation analysis revealed strong positive associations between individual phenolics and DPPH radical scavenging activity, particularly for *p*-coumaric acid ($r = 0.999$), procyanidin-B3 ($r = 0.987$), vanillic acid ($r = 0.964$), caffeic acid ($r = 0.953$), and ferulic acid ($r = 0.949$) (Figure S1). These compounds, characterized by hydroxylated aromatic rings and conjugated double bonds, exhibit high redox potential and electron-donating capacity, enabling them to effectively neutralize reactive oxygen species and stabilize free radicals (Platzer et al., 2022; Yamauchi et al., 2024). Similar relationships have been described in cereal-based matrices, where ferulic and vanillic acids dominate the antioxidant contribution, while caffeic and protocatechuic acids enhance hydrogen-atom transfer mechanisms (Ma et al., 2021). The strong linear association observed here demonstrates that the higher antioxidant capacity of BSD-B3 was not merely a function of its higher total phenolic content but rather the selective enrichment of structurally active, bioaccessible phenolics generated through bran addition and sourdough biotransformation. Compared to baker's yeast *Tritordeum* bread, sourdough breads showed lower levels of phytic acid and RFO, consistent with previous observations in sourdough and bran-based breads (Pontonio et al., 2020; Shao et al., 2025). Bran addition, whether through direct incorporation or fermentation, further enhanced DF, with BSD-B3 containing the highest TDF, including both SDF and IDF (Pontonio et al., 2020). Among DF components, β -glucan and AX are particularly relevant due to their health-promoting effects and contribution to cereal bran functionality (Coda et al., 2015). In our study, the highest levels of both were observed in bran-containing breads (TSD-B2 and BSD-B3). Importantly, these breads met the threshold of ≥ 3 g/100 g required by EC Regulation (EC No 1924/2006) to qualify as a "source of fiber." Bran-based sourdough used in breadmaking promoted proteolysis, reflected in higher TFAA than in baker's yeast bread, with BSD-B3 showing higher concentrations of essential amino acids. This effect results from the combined action of endogenous proteases and LAB peptidases, supported by the amino acid contribution of bran itself (Wang and Wang, 2024). Bran-containing sourdough breads also accumulated more GABA,

consistent with glutamate decarboxylation activity during fermentation (Rizzello et al., 2010). Sourdough fermentation enhanced IVPD compared to BYB, but bran addition caused a slight reduction, likely due to lignified non-digestible cell walls (Rizzello et al., 2012). Fermentation mitigated this limitation, as shown by higher proteolytic activity in BSD-B3, while other studies reported further improvement when exogenous xylanase was applied (Pontonio et al., 2020). Interestingly, TSD-B1 showed slightly higher IVPD than the bran-enriched bread. This may be explained by the lower degree of protein-fiber interactions, which can restrict enzyme access to cleavage sites (Li et al., 2024a), and by a reduced formation of Maillard-derived crosslinks that can block lysine residues and decrease digestibility during baking (Mildner-Szkudlarz et al., 2023; Qi et al., 2025).

In addition, sourdough fermentation and bran incorporation lowered the *pGI* of breads compared to the control, with the strongest effect in BSD-B3, reflecting both DF-mediated modulation of starch properties (Poutanen et al., 2014; Rizzello et al., 2017) and sourdough acidification, which delays starch digestion (Coda et al., 2015). The lower *pGI* in BSD-B3 is likely associated with slower starch hydrolysis induced by the bran matrix, where higher water binding and a denser crumb structure limit starch swelling and amylase access, while fermentation-driven changes further reinforce these effects (Renzetti et al., 2021; Rizzello et al., 2012). Moreover, starch protein and phenolic interactions formed during sourdough processing may further hinder enzymatic degradation, resulting in a lower hydrolysis rate and, consequently, a reduced estimated glycemic response (Fois et al., 2021).

Textural and color analyses highlighted the influence of bran and sourdough fermentation on bread quality. Specific volume, a key indicator of bread quality, differed between breads containing fermented and non-fermented bran. Bran-based sourdough breads showed higher volume than those with non-fermented bran, likely due to LAB acidification enhancing gluten's ability to retain CO₂ and improve dough expansion (Hanis-Syazwani et al., 2018). Bran addition also increased firmness compared to baker's yeast and 100 % *Tritordeum* sourdough breads, although fermented bran-based sourdough was less firm than breads with non-fermented bran. Similar effects of bran on firmness have been reported elsewhere (Hanis-Syazwani et al., 2018). As expected, bran incorporation also darkened crumb color, while bread made from 100 % *Tritordeum* flour retained a more yellowish hue due to its higher carotenoid content.

The human gut microbiome is dynamic and responsive to dietary inputs, with composition and metabolic activity closely linked to host health (Hou et al., 2022). We adopted the *in vitro* SHIME[®] model to evaluate whether the compositional differences among *Tritordeum* breads translate into physiologically relevant effects. The SHIME[®] set up was restricted to the proximal colon compartment since this region is the primary site for early degradation of fermentable substrates (Nogal et al., 2021); focusing on it enabled us to resolve short-timescale dynamics in response to the fiber- and phenolic-rich formulations (Mosele et al., 2015). Across treatments, acetate was the dominant SCFA and peaked at T1, in line with rapid saccharolytic activity typical of the proximal colon. The higher early acetate in TSD-B1 is compatible with its greater availability of rapidly fermentable carbohydrates (Facchin et al., 2024), whereas the later maximum in BSD-B3 at T2 reflects the slower degradation of complex fibers such as arabinoxylan (de Vries et al., 2016; Ivarsson et al., 2014). Propionate peaked at T2, particularly in TSD-B1 and BYB. Although *Bacteroides*, one of the main propionate producer in the colon (den Besten et al., 2013), declined sharply under all treatments, the dominance of *Bifidobacterium* suggests that cross-feeding between saccharolytic taxa and secondary fermenters, rather than direct *Bifidobacterium* metabolism, drove the observed increase (Wang et al., 2020).

Butyrate concentration decreased over time in all treatments; however, BSD-B3 consistently sustained higher concentrations than the other breads at each time point. This outcome indicates that the supply of suitable fermentation intermediates and complex carbohydrate

substrates supported butyrate formation despite a reduction in the relative abundance of canonical butyrogenic genera, consistent with the functional redundancy of butyrate-producing pathways in complex gut consortia (Esquivel-Elizondo et al., 2017; Zhao et al., 2024). Lactate accumulation was most pronounced in BSD-B3 at T1 and T2, consistent with its higher lactic acid content and fermentable fiber profile. The concomitant enrichment of *Acidaminococcus* and *Megasphaera*, both known lactate utilizers, suggests that BSD-B3 stimulated metabolic cross-feeding loops (Wang et al., 2020). In addition, *Weissella* expanded in parallel with lactate and acetate, in agreement with its known role as a heterofermentative LAB that contributes to early carbohydrate breakdown and acid production (Fusco et al., 2015). *Bifidobacterium* showed strong positive associations with both acetate and lactate, reflecting its prominent saccharolytic activity across treatments (Louis et al., 2022). *Collinsella* also correlated with SCFA levels in BSD-B3, likely reflecting an indirect role in supplying intermediates such as acetate and lactate that can be converted downstream into butyrate (Rajilic-Stojanovic and de Vos, 2014). The subsequent decline of lactate by W1 confirms efficient metabolic turnover rather than accumulation. The integrated summary of SCFA production and the genera significantly associated with treatment or time (Figure S2) further supports these observations. In particular, increases in genera linked to saccharolytic activity and butyrate formation (e.g., *Blautia*, *Butyricoccus*) corresponded with the higher butyrate concentrations observed during BSD-B3 treatment, whereas *Acidaminococcus* and *Bifidobacterium* displayed treatment-specific fluctuations consistent with differential substrate utilization.

Microbiota composition shifted markedly over time, with *Bifidobacterium* remaining the dominant genus throughout. Its strongest expansion occurred in TSD-B1 and BYB, which is consistent with evidence that fermentable carbohydrates stimulate bifidogenic growth in the colon (De Vuyst and Leroy, 2011). In contrast, BSD-B3 was characterized by higher relative abundances of *Acidaminococcus* and *Collinsella*. *Acidaminococcus* has been linked to amino acid metabolism and lactate utilization, often in association with cross-feeding to other taxa (Louis and Flint, 2017), whereas *Collinsella* is associated with the degradation of complex carbohydrates and the production of fermentation intermediates such as acetate and lactate (Tas and Ulgen, 2023). Both taxa therefore may have contributed indirectly to the SCFA dynamics observed in BSD-B3. The consistent decline of *Bacteroides* and *Blautia* across treatments reflects a broader restructuring of the community in response to *Tritordeum* breads, though the specific drivers of this shift remain unclear. *Enterobacter*, which increased from T1 onwards, was positively associated with propionate but negatively with butyrate, suggesting ecological trade-offs in carbon fluxes during later fermentation stages (Henson and Phalak, 2018).

4. Conclusion

This study is the first to evaluate sourdough fermentation of *Tritordeum*-bran composite with selected LAB and yeast strains. Fermentation markedly enhanced the biochemical profile of *Tritordeum*-bran sourdough consequently improving DF, antioxidant activity, free amino acids, phenolic compounds, and lowering the *pGI* of breads. Through the SHIME[®] experiment, BSD-B3 favored butyrate release and distinct microbial restructuring, while TSD-B1 and BYB mainly stimulated acetate- and propionate-linked bifidogenic activity. These findings indicate that *Tritordeum* sourdough breads offer a sustainable approach to valorize bran while improving overall bread quality and selectively shaping gut microbial metabolism. Future work should extend these findings to consumer studies, assessing sensory acceptance and preference of *Tritordeum*-based baked goods, while *in vitro* microbial observations should be validated through *in vivo* studies to better elucidate their potential effects on the gut.

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Ethics approval statement

The collection and use of human fecal samples were conducted in accordance with the Declaration of Helsinki and were approved by the Ethics Committee of the Free University of Bozen on 17 July 2019.

CRediT authorship contribution statement

Bilal Sajid Mushtaq: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Olga Nikoloudaki:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Methodology, Investigation. **Martina Ben:** Writing – original draft, Investigation, Formal analysis, Data curation. **Kashika Arora:** Formal analysis, Data curation. **Ali Zein Alabiden Tlais:** Writing – review & editing, Methodology, Data curation. **Andrea Polo:** Writing – review & editing, Methodology. **Raffaella Di Cagno:** Writing – review & editing. **Marco Gobetti:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.fufo.2025.100874](https://doi.org/10.1016/j.fufo.2025.100874).

Data availability

The sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1332428. Associated BioSample and SRA accession numbers for each replicate and condition are provided within the BioProject record.

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