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Sourdough performances of the golden cereal *Tritordeum*: Dynamics of microbial ecology, biochemical and nutritional features

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ABSTRACT

The novel cereal '*Tritordeum*' was employed in sourdough fermentation for bread making using a traditional backslopping procedure over 10 days. Culture-dependent and culture-independent approaches were used to characterize microbial ecology during sourdough preparation and propagation. Sourdough reached the highest microbial diversity after three days of propagation. Microbial diversity decreased as sourdough reached maturity (day 5). Microbiota dominance shifted from *Weissella* to *Lactiplantibacillus* genera after 5 days of propagation. Lactic acid bacteria (LAB) showed a constant increase throughout the propagations starting from 3.9 ± 0.24 log CFU g^{-1} on day 0 up to 8.0 ± 0.39 log CFU g^{-1} on day 5. *Weissella confusa/cibaria* and *Weissella paramesenteroides* were the most prevalent LAB species until day 5 of propagation, while *Lactiplantibacillus plantarum* was the most prevalent thereafter. Yeasts were present in low cell density (2.0 ± 0.11 log CFU g^{-1}) until the fourth backslopping (day 4) and then gradually increased until day 10 (5.0 ± 0.29 log CFU g^{-1}), with *Saccharomyces cerevisiae* being the most prevalent and dominant species. Lactic and acetic acid concentrations increased throughout *Tritordeum* sourdough propagations, indicative of a proportional decrease of fermentation quotient (lactic acid/acetic acid) from 13.54 ± 1.29 to 4.08 ± 0.15. Utilization of glucose, fructose and sucrose was observed, followed a progressive increase in mannitol concentrations beginning from day 4. The nutritional potential (total phenol content, antioxidant activity, dietary fiber content and total free amino acids) remained elevated during sourdough propagations. Antinutritional factors (phytic acid and raffinose) were reduced to minimal concentrations by day 10. Finally, texture analysis of *Tritordeum* sourdough bread was demonstrated to have better cohesiveness, resilience and firmness compared to baker's yeast bread, confirming its potential to improve functionality and use in sourdough biotechnology.

1. Introduction

Interest in sourdough fermentation is thriving and there is a continuous introduction of new and alternative flours from cereals, pseudo-cereals, legumes and food by-products (Cantatore et al., 2019; Montemurro et al., 2019; Rizzello et al., 2014). Moreover, with the world's population continuing to grow and the resulting need to attain sustainable nutrition by 2050, there is currently increasing interest in the exploitation of well-adapted, climate-resilient and nutritious crops. With the aim of fitting into such a scenario, cereal breeders have concentrated their efforts on developing interspecies hybrids to obtain new cereals with higher phytochemical content and better agronomic performance and technological qualities. Among these new hybrid cereals, hexaploid *Tritordeum* has emerged as a leading candidate with significant potential to impact on the sustainability of baked goods. *Tritordeum*, often called the golden cereal, is the amphidiploid cereal resulting from the cross between a South American wild barley (*Hordeum chilense* Roem. et Schultz.) and a cultivated durum wheat (*Triticum turgidum* ssp. *durum* Desf.) grown mainly in the Mediterranean region (Martín et al., 1999). *Tritordeum* brings together the nutritional content of durum wheat and barley (Suchowilska et al., 2021) to become as one of the most sustainable food ingredients, which can withstand climate change without impacting the environment ("Tritordeum," 2018). Recently, *Tritordeum* was introduced in the European market as novel

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cereal and also proposed as a suitable cereal for the reduction of gluten sensitivity compared to wheat (Vaquero et al., 2018). *Tritordeum* flour has a low gluten content and this can be further reduced through gluten degradation during sourdough fermentation (Di Cagno et al., 2010; Dingeo et al., 2020). *Tritordeum* is also rich in carotenoids, responsible for its strong yellow color (Paznocht et al., 2018), tocoferols (Lachman et al., 2018) and phenolic acids. These compounds are all antioxidants and have been shown to influence the pigmentation of *Tritordeum* grains and impart health benefits, e.g., by reducing cholesterol levels in the serum (Lachman et al., 2018). Similarly, *Tritordeum* contains β-glucans, complex carbohydrates which have been proposed as prebiotics and hold EFSA and USDA health claims for reducing cardiovascular disease risk ("CFR - Code of Federal Regulations Title 21," 2021; "Oat beta glucan and lowering blood cholesterol|EFSA," 2010; (Fernandez-Julia et al., 2021)). Therefore, *Tritordeum* has considerable potential as an ingredient for functional food production and its health promoting features could be enhanced by sourdough fermentation (Gobbetti et al., 2020). Despite the extensive characterization of *Tritordeum* at the crop level (Lachman et al., 2018; Paznocht et al., 2018), or its use for making regular baker's yeast bread, the application of this cereal in sourdough fermentation for bread making has not yet been described. Consequently, efforts should be taken to optimize the use of *Tritordeum* flour for making high nutritional value bread with increased consumers' acceptability. However, the combination of *Tritordeum* and sourdough as leavening agent for making bread allows both to investigate how the cereal affects the evolution of the microbiota during fermentation and to explore the impact on the nutritional and sensory properties of the resulting bread. The use of the *Tritordeum* to make bread with commercial baker's yeast would only have made it possible to investigate the effect of the cereal alone.

In this study, *Tritordeum* flour (supplied by Molino Rachello, Italy) was used in traditional type I sourdough-type biotechnology employing the backslopping procedure. Traditional sourdoughs are prepared by multiple steps of fermentation (backslopping). First, dough composed of flour and water is spontaneously fermented. Subsequently, this fermented dough is used as an inoculum for fermenting newly prepared dough, which, in turn, will be used as inoculum for a subsequent step of fermentation (Arora et al., 2021), achieving a stable consortium of yeasts and lactic acid bacteria with leavening and acidifying capacity. The metabolic activity of sourdough microbiota and its structure deeply affect the characteristics of the mature sourdough (Ercolini et al., 2013; Gänzle and Ripari, 2016). Focusing on this perspective, we investigated the evolution dynamics of microbiological, biochemical, and nutritional properties during the preparation and propagation of *Tritordeum* sourdough by traditional fermentation (type I sourdough) over 10 days in order to assess its potential use in bread making.

2. Materials and methods

2.1. Flour

Tritordeum flour (organic) was purchased from the mills of Molino Rachello (Veneto, Italy). The gross composition was as follows: moisture, 11 ± 1.0%, protein (N × 5.7), 12 ± 0.8% of dry matter (d.m.); total carbohydrates, 83 ± 2.5%, of d.m.; dietary fiber, 12.5 ± 0.5% of d.m., and fat 2.5 ± 0.15 of d.m. Three different batches of flour were pooled and used to prepare the sourdough.

2.2. Dough preparation and sourdough propagation of Tritordeum flour

Preparation of dough and propagation of sourdough was performed by traditional protocol (Di Cagno et al., 2014; Minervini et al., 2012), without the addition of starter cultures or baker's yeast. Dough preparation was performed as follows: *Tritordeum* flour (187.5 g) and tap water (112.5 mL) were used to produce 300 g of dough (dough yield [$DY = \left(\frac{dough\ weight}{flour\ weight}\right)$*100], 160) with a continuous high-speed mixer (60 ×*g*, dough mixing time of 5 min) (Esmach (Type SPI 30 F), Italy). This preparation yields dough prior to fermentation and before becoming sourdough. Each sourdough was subjected to fermentation (propagation) at 25 °C for 5 h daily. The only exception was the first fermentation, which lasted 8 h, according to traditional protocol (Minervini et al., 2012). Between each daily fermentation, sourdoughs were stored at 4 °C for ca. 16 h. Sourdough propagation was according to the backslopping (refreshment) procedure, where the sourdough from the day before was used as the starter (25% [w/w] of inoculum) to ferment a new mixture of flour (140.62 g) and tap water (84.38 mL), having a dough yield of 160. Leavening power is defined as the rise in dough volume due to the entrapment of carbon dioxide released by yeasts within the gluten matrix during sourdough fermentation. The dough was placed in a 500 mL beaker up to the mark of 100 mL and the increase (fold change) in volume was measured at the end of each fermentation. Sourdoughs were daily propagated for 10 days, and samples were taken after 0 (dough), 1, 2, 5, 7 and 10 (sourdough) days of propagation. Sourdoughs were cooled down to 4 °C and analyzed within 2 h after collection. Preparation and propagation of sourdough was carried out in triplicates.

2.3. Microbiological characterization

Ten grams of dough sample, on day 0 and on each day after fermentation, was homogenized with 90 mL of sterile physiological (0.9% [w/v] NaCl) solution. *Enterobacteriaceae* were enumerated on Violet Red Bile Dextrose (VRBD) agar medium (Merck, Germany) at 37 °C for 24 h. Lactic acid bacteria were counted at 30 °C for 48 h under anaerobiosis using modified MRS agar (Oxoid, Basingstoke, Hampshire, United Kingdom) medium, prepared by the addition of 0.5% [w/v] maltose and 0.5% [w/v] yeast extract to standard MRS medium and supplemented with cycloheximide (0.1 g/L) (Sigma-Aldrich, USA). Yeasts were enumerated at 30 °C for 48 h on Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, Hampshire, United Kingdom) medium supplemented with chloramphenicol (0.1 g/L) (Sigma-Aldrich, USA).

2.4. Isolation, genotyping and identification of lactic acid bacteria and yeasts

At least 20 colonies of presumptive lactic acid bacteria at each timepoint were randomly selected from the plates containing the two highest sample dilutions. Gram-positive, catalase-negative, nonmotile rod and coccus isolates were cultivated in SDB broth at 30 °C for 24 h and restreaked onto the same agar medium. All isolates considered for further analysis were able to acidify the culture medium. Fifteen randomly selected colonies of yeasts at each timepoint from the highest plate dilutions were subcultured in SDA and restreaked onto the same agar media.

Genomic DNA from presumptive LAB and yeasts was extracted using DNeasy Blood & Tissue Kit (Qiagen, Italy). The cell lysis for bacterial cultures was performed using lysozyme (L6876, Sigma-Aldrich) while lyticase enzyme from *Arthrobacter luteus* (L2524, Sigma-Aldrich) was used for the lysis of yeasts. The biotyping of LAB isolates was performed as reported by (Di Cagno et al., 2014). Two oligonucleotides, M13m (5′-GAGGGTGGCGGTTC-3′) and Rp11 (5′-GAAACTCGCCAAG-3′) (35), were used singly in two series of amplifications for biotyping of yeast isolates. Randomly amplified polymorphic DNA (RAPD)-PCR profiles were acquired by the microchip electrophoresis system MCE-202 MultiNA (Shimadzu Italia Srl, Milano, Italy), as previously described (Minervini et al., 2016). The treeflap macro was used (http://www.sci.monash.edu.au/wsc/staff/walsh/treeflap.xls) to build the binary matrix for cluster analysis. The similarity of the electrophoretic profiles was assessed using Euclidean distance and hierarchical method (Unweighted Paired Group Mathematic Average, UPGMA), with R studio version 3.2.5.

Bacterial and yeast isolates were identified by partial sequencing of the 16S rRNA gene and an internal transcribed spacer (ITS), respectively. The primer pair, LpigF/LpigR (5′-TACGGGAGGCAGCAGTAG-3′ and 5′-CATGGTGTGACGGGCGGT-3′) (Sigma-Aldrich), was used for amplifying the 16S rRNA gene of lactic acid bacteria (De Angelis et al., 2006). While the primer pair NL-1/NL-4 (5′-GCATATCAA-TAAGCGGAGGAAAAG-3′ and 5′-GGTCCGTGTTTCAAGACGG-3′) (Sigma-Aldrich.) was used to amplify the D1/D2 domain of the 26S rRNA gene (Kurtzman and Robnett, 1998) for yeasts. Primers designed for the recA gene (Torriani et al., 2001) were also used to distinguish *Lactiplantibacillus plantarum*, *L. pentosus*, and *L. paraplantarum* species (formerly known as *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum*, respectively) (Zheng et al., 2020). PCR products were separated by electrophoresis. Identification was performed by comparing the sequences of each isolate with those reported in the Basic BLAST database (Altschul et al., 1997). Strains showing homology of at least 97% were considered to belong to the same species (Stackebrandt and Goebel, 1994).

2.5. Total microbial genomic DNA extraction

Total genomic DNA was extracted from *Tritordeum* dough prior to fermentation (D0), after the first fermentation (D1), and after 2 (D2), 5 (D5), 7 (D7) and 10 (D10) days of sourdough propagation. Samples were processed according to the following procedure in order to reduce the contamination with chloroplast DNA (Minervini et al., 2010): 8 g of sourdough sample was homogenized in 40 mL of sterile potassium phosphate buffer (PBS, 50 mM, pH 7.0) followed by centrifugation at 200 ×*g* for 5 min. The supernatant was then subjected to a second centrifugation step at 1500 ×*g* for 5 min at 4 °C, and a further centrifugation at 14,000 ×*g* for 15 min at 4 °C. The pellet obtained was subjected to total DNA extraction by FastDNA Soil Kit (FastDNA Spin Kit for Soil, MP Biomedicals, Italy). Quantification of total DNA was performed with Qubit™ dsDNA HS Assay Kit (Thermofisher Scientific, Italy). Three independent replicates of each sample were used for DNA extraction and pooled for amplicon generation.

2.6. Preparation of the MiSeq library

Three DNA samples corresponding to the three biological replicates for each dough and sourdough were used for 16S rRNA and internal transcribed spacer (ITS)-based bacterial and fungal diversity analysis, respectively. Primers targeting the variable region V3–V4 of 16S rRNA gene (*Escherichia coli* position 341–805, forward 341F: CCTACGGGNGGCWGCAG and reverse 806R: GACTACNVGGGTWTC-TAATCC) (Claesson et al., 2010) of the 16S rRNA gene were used for bacteria, while primers (forward ITS1: CTTGGTCATTTAGAGGAAGTAA and reverse ITS2: CTGCGTTCTTCATCGATGC) targeting the ITS1 region between 18S and 5.8S rRNA genes were used for fungi (Gardes and Bruns, 1993). Unique barcodes were attached to the forward primer for facilitating the differentiation of samples. Amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter) according to manufacturer's instructions, in order to prevent preferential sequencing of smallest amplicons, and DNA was quantified using the Quant-iT Pico-Green dsDNA kit (Invitrogen). Amplicons were mixed and combined in equimolar ratios, and the quality and purity of the library was evaluated with the High Sensitivity DNA Kit (Agilent, Palo Alto, CA, USA) by the Bioanalyzer 2100 (Agilent). Library preparation and pair-end sequencing were carried out at the Genomic Platform – Fondazione Edmund Mach (San Michele a/Adige, Trento, Italy) using the Illumina MiSeq system (Illumina, USA) according to standard laboratory procedures.

2.7. Illumina data analysis and sequences identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (QIIME2, version 2018.2). Sequences were quality filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative bacterial sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Katoh and Standley, 2013; Price et al., 2009). Alpha and beta diversity metrics were calculated using the core-diversity plugin within QIIME2 and emperor (Vázquez-Baeza et al., 2013). Alpha diversities were determined based on the number of observed operational taxonomic units (OTU), Chao1 and Shannon indices. These alpha diversity indices represent the species richness within each sample while Shannon index also indicates the evenness of the identified taxonomy. Beta-diversities were calculated using the Bray Curtis distance matrix in QIIME2. Beta-diversity distance matrix indicates differences in taxa composition among samples based on either presence-absence or quantitative species abundance data. Output matrix was ordinated using principal coordinate analysis (PCoA) and visualized using EMPeror (Vázquez-Baeza et al., 2013). Bacteria taxonomic and compositional analyses were carried out by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A naive Bayes taxonomy classifier was trained on the Silva (Yilmaz et al., 2014) r132 reference sequences (clustered at 99% similarity) using q2-feature-classifier's fit-classifier-naive-bayes method, trimmed to the V3-V4 region of 16S rDNA, and applied to paired-end sequence reads to generate taxonomy tables. Fungi sequences were classified to the species-level with a 97 or 99% threshold (based on which is more accurate for certain lineages of fungi) by using UNITE v.8.0 Dynamic Classifier (Nilsson et al., 2019). Number of sequences analyzed before and after filtering, observed OTUs, Chao1 and Shannon indices for 16S rRNA gene and ITS amplification were subjected to one-way ANOVA; pair comparison of treatment means was obtained by Tukey's procedure at $P < 0.05$, using the statistical software R 3.6.1 (R Core Team, 2019).

2.8. Nucleotide sequence accession number

The sequences are available in the Sequence Read Archive of NCBI (accession number PRJNA791193).

2.9. Water-soluble extracts (WSE) and methanol-soluble extracts (MSE) preparation

Water-soluble extracts from dough (D0) or sourdough samples after 1 (D1), 2 (D2), 5 (D5), 7 (D7) and 10 (D10) days of propagation were prepared according to the method originally described by Osborne (1907) and modified by Weiss et al. (1993). Briefly, 2 g of sample was suspended in 8 mL of 50 mM Tris–HCl (pH 8.8), held at 4 °C for 1 h, vortexing at 15 min intervals, and centrifuged at 20,000 ×*g* for 20 min. The supernatants were used for analyses. Similarly, 2 g of samples were mixed with 8 mL of 80% methanol to get MSE. The mixture was stirred in a shaker for 30 min and centrifuged at 4600 ×*g* for 20 min. The supernatants (or MSE) were transferred into tubes without the pellet and stored at 4 °C before analysis.

2.10. Biochemical characterization

The values of pH were determined by a pH meter (SensION™+ PH3, Hach, Italy) with a food penetration probe. Total titratable acidity (TTA) was measured on 10 g of sample, which was homogenized with 90 mL of distilled water for 3 min in a bag mixer (400P; Interscience, St Nom, France), and expressed as the amount (mL) of 0.1 M NaOH needed to achieve the pH of 8.3 units. WSEs from dough and sourdoughs were used for the determination of lactic and acetic acids by high-performance liquid chromatography (HPLC) equipped with an Aminex HPX-87H column (ion exclusion, Biorad, Richmond, CA), and an UV detector

operating at 210 nm. Elution was at 70 °C, with a flow rate of 0.6 mL/min, using H_2SO_4 5 mM as mobile phase (Tlais et al., 2020). The quotient of fermentation (FQ) was determined as the molar ratio between D, L-lactic and acetic acids.

WSEs were also used for the determination of carbohydrates using a Spherisorb column (4.6 × 250, Waters, USA) and an HPLC equipped with a refractive index detector (RI-101, Perkin Elmer, USA). A solution of acetonitrile/water (80:20 v/v) was used as mobile phase (flow, 1 mL/min). The identification of the sugars and the calibration curves were obtained using commercial standards of glucose, fructose, sucrose and mannitol (Sigma Aldrich, USA).

Ten grams of dough (0 day) and sourdoughs (1, 2, 5, 7 and 10 days) were diluted with 40 mL of Tris HCl 50 mM (pH 8.8), stirred continuously for 1 h at 4 °C and then centrifuged (10,000 ×*g*, 10 min at 4 °C). Total free amino acids were determined in the supernatant by Cd-ninhydrin spectrophotometric method (Doi et al., 1981).

2.11. Nutritional characterization

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was evaluated on MSE of dough and sourdough samples as described by Yu et al. (2004). The scavenging activity was expressed as follows: DPPH scavenging activity (%) = [(blank absorbance − sample absorbance) / blank absorbance] × 100. The absorbance value was compared with that of 75-ppm butylated hydroxytoluene (BHT) as the antioxidant reference. MSE of samples were also used for the determination of total phenols according to the Folin-Ciocalteu method (Singleton and Rossi, 1965). An aliquot of extracts of 20 μL was added to 1.58 mL distilled water in spectrophotometer cuvettes, followed by 100 μL of Folin-Ciocalteu reagent was added. The solution was mixed and allowed to equilibrate. After 8 min, 300 μL of sodium carbonate solution was added. The mixture was shaken at 40 °C for 30 min. Then, the absorbance was measured at 740 nm using UV–Visible spectrophotometer (Shimadzu PharmaSpec UV 1800 Double Beam UV–Vis Spectrophotometer, Japan). Data were expressed as gallic acid equivalents. Total, soluble and insoluble dietary fiber content at 0, 5 and 10 days was determined according to the AOCC Official Method 991.43. Briefly, dough and sourdoughs were first subjected to sequential enzymatic digestion with heat stable α-amylase, protease and amyloglucosidase for the removal of starch and proteins. Dietary fibers were purified and deposited on filtering crucibles (Velp Scientifica, Italy) containing Celite (545 AW, Sigma Chemical Co., Italy) using vacuum filtration system (Velp Scientifica, Italy) followed by washing steps. The insoluble dietary fiber (IDF) was deposited on the crucibles by filtration, while the soluble dietary fiber (SDF) was collected as filtrate. SDF was subjected to precipitation with 4 volumes of 95% ethanol. The precipitated fibers were re-filtered under vacuum on fresh celite crucibles. All the crucibles with IDF and SDF were washed with 78% ethanol and acetone followed by overnight drying in the oven at 105 °C. The weight of the crucibles was measured before and after drying to calculate the SDF and IDF values, respectively. Ash was determined by treating the processed samples in crucibles at 525 °C in a muffle furnace. The SDF and IDF values were corrected for protein, ash and blank while the total dietary fiber (TDF) values were determined as the sum of IDF and SDF.

Phytic acid concentrations were determined using Phytic Acid (Phytate)/Total Phosphorus kit (Megazyme International, Ireland), which is the modified and improved protocol of the AOAC official method 986.11. Briefly, 1 g of lyophilized dough (day 0) and sourdough (days 5 and 10) samples were digested with 20 mL of 0.66 M HCl in 50 mL tubes overnight at room temperature. The digested samples were centrifuged at high speed (13,000 rpm, 10 min) to collect the supernatant, which was further neutralized by the addition of 0.75 M NaOH. The neutralized extracts were further processed to estimate the free and total phosphorus content using the phosphorus calibration curve according to manufacturer's instructions. The phosphorus determination was used as a measure to estimate the phytic acid concentrations using the mathematical formula provided in the instruction manual, assuming that the amount of phosphorus is exclusively released from phytic acid (28.2%).

Raffinose concentration was determined using the Raffinose/D-Galactose kit (Megazyme International, Ireland), according to the manufacturer's instructions. Raffinose was hydrolyzed to D-galactose and sucrose by α-galactosidase. This enzyme also hydrolyses other α-galactosides such as stachyose, verbascose and galactinol, if present. The α-anomeric form was converted into β-D-galactose by galactose mutarotase. β-D-galactose was oxidized by NAD^+ to D-galactonic acid, in the presence of β-galactose dehydrogenase. The amount of NADH, measured through the increase of absorbance at 340 nm, was stoichiometric with the amount of D-galactose released.

2.12. Bread making and texture profile analysis (TPA)

Breads were prepared according to typical Italian bread recipe having dough yield of 160 at the Bakery Insperience pilot plant of the Micro4Food lab (Libera Universitá di Bolzano, Italy). The bread formulas were as follows: (1) *Tritordeum* baker's yeast bread (BYB) made with 250 g *Tritordeum* flour, 150 g tap water and 1.5% (w/w) of commercial baker's yeast; and (2) *Tritordeum* sourdough bread (SDB) made with 175 g *Tritordeum* flour, 105 g tap water and 120 g *Tritordeum* sourdough (30%, [w/w] of total dough). A continuous high-speed mixer (60 ×g, dough mixing time 5 min) was used to prepare the doughs. Fermentation of doughs was allowed at 30 °C for 2.5 h. All breads were baked at 230 °C for 35 min (Omega 2, Bongard, Italy). Fermentations were carried out in triplicates and each bread was analyzed twice. Instrumental texture profile analysis (TPA) was performed by TVT 6700 Texture Analyzer, using a 25 mm cylindrical probe (probe P-CY25S). The test mode settings were as follows: test speed 1 mm/s, 20% compression distance and two-compression cycle (TVT method 01-03.01). TPA was carried out using TexCalc 5 software, which measured specific volume, height, width, depth, and area of loaves.

2.13. Statistical analysis

Three batches of *Tritordeum* flour were used in this study. From each batch, three different sourdoughs (total of 9 sourdoughs) were prepared and propagated and analyzed in triplicates ($n = 3$). Data were subjected to one-way ANOVA and the pair comparison of treatment means was obtained by Tukey's post-hoc analysis at $P < 0.05$, using the statistical software Statistica 8.0 (StatSoft Inc., USA). A Pearson correlation (r) analysis was used to determine significant relationships ($P < 0.05$) between the microbial composition of dough and sourdough samples and their biochemical and nutritional profile using the software R version 3.6.1 (R Core Team, 2019).

3. Results

3.1. Microbial counts and acidification during sourdough preparation and propagation

After the first day of fermentation (8 h at 25 °C), cell density of presumptive LAB increased significantly from 3.9 ± 0.24 to 5.0 ± 0.36 log CFU g^{-1} ($P < 0.05$) and then continued to increase up to 8.0 ± 0.39 log CFU g^{-1} until day 5 of sourdough propagation (Fig. 1). This cell density was maintained within half a log cycle during subsequent propagation. Compared to presumptive LAB, the dough contained a lower (2.0 ± 0.11 log CFU g^{-1}) number of yeasts (Fig. 1), which significantly increased from day 5 onwards (5.0 ± 0.29 log CFU g^{-1}, $P < 0.05$) and remained constant thereafter. The ratio between LAB and yeasts stabilized to ca. 1000:1 after 5 days of propagation. *Enterobacteriaceae* enumerated in the dough significantly increased after 1 day (4.0 ± 0.31 log CFU g^{-1}, $P < 0.05$), but decreased from day 4 onwards and went below the detection limit of the culture-dependent technique

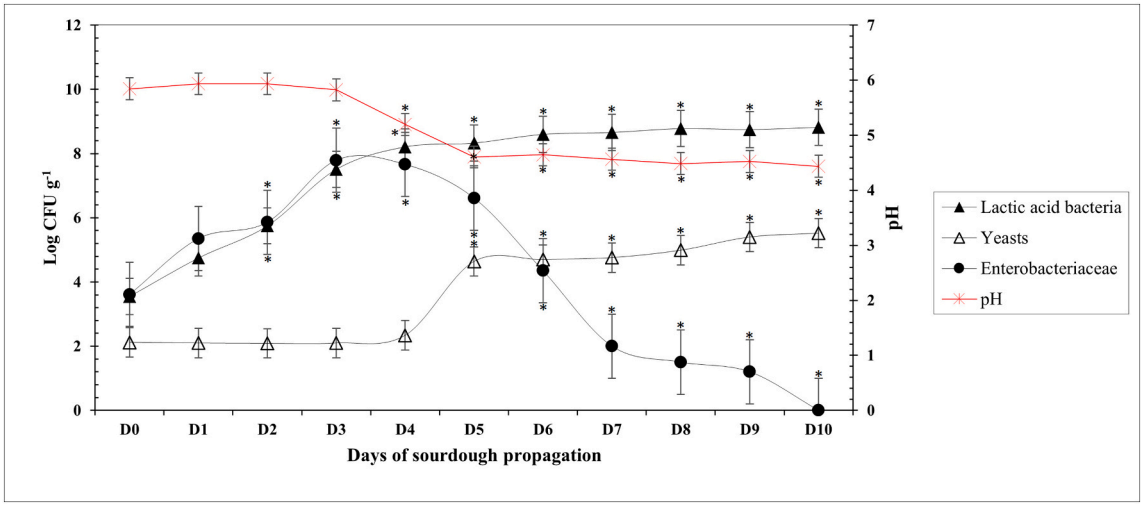

Fig. 1. Cell densities (log CFU g^{-1}) of presumptive lactic acid bacteria, yeasts, and *Enterobacteriaceae* and kinetic of acidification during *Tritordeum* sourdough preparation and propagation. Sourdough was daily propagated for 10 days; *x*-axis indicates the days of propagation (D0 to D10). Day 0 (D0) represents dough prior to fermentation and before becoming sourdough. Data are the means of three independent experiments ± standard deviations ($n = 3$) analyzed in duplicates and submitted to one-way analysis of variance (ANOVA). *$P < 0.05$ D1-D10 vs D0.

by the end of sourdough propagation (Fig. 1). The pH values of sourdough reflected the LAB enumeration. Before the first fermentation, the pH value of dough was 5.90 ± 0.13, pH significantly ($P < 0.05$) decreased from day 3 of propagation and stabilized at 4.3 ± 0.21 from day 5 onwards (Fig. 1).

3.2. Evolution of microbiota composition, as determined by culture-dependent analysis, during sourdough preparation and propagation

Gram-positive, catalase-negative, non-motile cocci and rods, able to acidify mMRS broth, randomly isolated from the highest plate dilutions of each time-point, were subjected to RAPD-PCR analysis. At a similarity level of 85%, 200 isolates were gathered into six clusters (Fig. S1). Partial sequencing of the 16S rRNA gene identified isolates representative of each cluster. Six species of LAB were identified and their succession over time was shown in Fig. 2. The highest prevalence was observed for *Weissella confusa* (69 ± 9.8% isolates) and *W. confusa/cibaria* (15 ± 5.0%). These species were found throughout the propagation. *Weissella paramesenteroides* (10 ± 4.5%) inhabited the dough and persisted up to 5 days of propagation. *W. paramesenteroides* then appeared to be replaced by *L. plantarum* (56 ± 12.5%), *Latilactobacillus curvatus* (17 ± 3.8%) and *Leuconostoc mesenteroides* (9 ± 7.0%). One-hundred and fifty yeast isolates from the highest dilution plate for each time-point were also subjected to RAPD-PCR analysis. Isolates were grouped into six clusters with a similarity level of 85%, (Fig. S1). One representative isolate for each cluster was identified by partial sequencing of the 28S rRNA gene. 90% of total isolates were identified as *Saccharomyces cerevisiae*, 7% as *Clavispora lusitaniae*, and 3% as *Pichia kudriavzevii*. *S. cerevisiae* was the dominant isolate throughout 10 days of sourdough propagation, while *C. lusitaniae* appeared at its highest dominance after 4 days and persisted up to 9 days. Finally, *P. kudriavzevii* reached his highest prevalence during the last days of propagation (Fig. 2).

3.3. Evolution of microbiota during sourdough preparation and propagation as estimated by high throughput sequencing

Total (2,316,647 and 2,173,066) and quality-trimmed (1,741,336 and 1,563,668) sequences for 16S rRNA gene and ITS1 region explained the entire bacterial and fungal diversity of the *Tritordeum* dough and sourdoughs. Number of observed operational taxonomic units (OTU), Chao1 and Shannon indices are reported in Table S1. The highest bacterial diversity was found for dough (D0) and sourdoughs after 1 (D1) and 2 (D2) days of fermentation, as shown by a significant increase in Observed OTUs, Chao1 and Shannon indices of α-diversity. After 5 days (D5) of propagation, the diversity progressively decreased over time in line with selection of a fermentative microbiota (Table S1). A similar trend was found for yeast diversity, except for a slight but significant ($P < 0.05$) increase after 5 (D5), 7 (D7) and 10 (D10) days of propagation. Microbial diversity was also evaluated using the Bray Curtis beta diversity metric and represented on a principal coordinate analysis (PCoA) (Fig. S2A–B). PCoA for bacteria and yeasts differentiated samples based on the fermentation and propagation time. Indeed, dough before fermentation (D0), as well as sourdoughs after 1 (D1) and 2 (D2) days of fermentation showed clustering both according to 16S rRNA gene and ITS PCoA; D5 and D7 clustered in the middle, and D10-sourdough on the opposite side (Fig. S2).

The bacterial phyla and their relative abundances (%) varied depending on number of propagations (Fig. 3A). Proteobacteria was the most abundant phylum in the dough before fermentation (D0), after the first day of fermentation (D1) and after 2 days of propagation (D2), whereas Firmicutes dominated after 5 (D5), 7 (D7) and 10 (D10) days propagation. (Fig. 3A). The bacterial taxonomic composition at genus level is shown in Fig. 3B and Fig. S3A. Based on the identified bacteria and their relative abundances, dough and sourdoughs were grouped in two clusters (I and II). Cluster I included all doughs before fermentation (D0), sourdoughs after 1 day fermentation (D1) and 2 days propagation (D2), whereas cluster II included all sourdoughs after 5 (D5), 7 (D7) and 10 days (D10) propagation. Clustering was consistent with the PCoA (Fig. S2A). *Pantoea* (45.1 ± 18.0%) and other *Enterobacteriaceae* (17.4 ± 14.0%) dominated the dough at D0, followed by *Lactiplantibacillus* (7.7 ± 5.5%) and *Erwinia* (10.8 ± 4.9%). The bacterial composition remained quite stable over D1 and D2 of propagation. After 5 (D5), 7 (D7) and 10 (D10) days of propagation, *Lactiplantibacillus* became the dominant genus (73.6 ± 43.7, 86.0 ± 24.0, and 91.9 ± 13.9%, respectively for D5, D7 and D10), while *Pantoea*, other *Enterobacteriaceae* and *Erwinia* completely disappeared (Fig. 3B and Fig. S3A). *Weissella* was detected in dough (D0) (1.3 ± 0.39%) and in sourdough after 1 day fermentation (D1) and 2 days propagation (D2) (1.7 ± 0.8 and 4.2 ± 2.5%, respectively for D1 and D2), then it reached the highest relative abundance after 5 days propagation (D5) (21.1 ± 16.4%) and decreased again after 7 (D7) and 10 (D10) days propagation (9.0 ± 5.6 and 2.2 ± 3.8%, respectively for D7 and D10). *Enterobacter*, *Kosakonia*, *Pantoea*, and *Raoultella* – *Serratia*, *Clostridium*, *Paenibacillus*, *Xanthomonas*,

Pseudomonas, *Luteibacter*, *Curtrobacterium*, *Burkholderiaceae*, *Acinetobacter*, *Sanguibacter*, *Staphylococcus* and *Sphingomonas*, which were present in the dough (D0) at low relative abundance (<2.0%), disappeared after 10 days of propagation.

Sequences from ITS region assigned to yeast phyla and their relative abundances (%) were mostly stable throughout the days of propagations (Fig. 3C). Dough (D0) was dominated by Ascomycota (87.5 ± 0.9%), with low relative abundances of Basidiomycota (2.9 ± 1.1%). Yeast taxonomic composition at genus level grouped dough and sourdoughs in three clusters (I, II and III) (Fig. S3B), consistently with the PCoA (Fig. S2B). Cluster I included doughs prior fermentation (D0), and after 1 (D1) and 2 (D2) days of sourdough propagation, cluster II included D5 and D7 sourdoughs, and cluster III included sourdough after 10 days (D10) of propagation. *Alternaria* and *Microdochium* dominated dough prior to fermentation (25.7 ± 0.7% and 18.3 ± 0.2%, respectively) followed by *Nectriaceae* (13.4 ± 1.0%) and *Epicoccum* (9.7 ± 0.9%), whereas *Saccharomyces* was present at very low relative abundance (0.04 ± 0.02%). The yeast profile was mostly stable after 1 day fermentation (D1) and 2 days propagation (D2), while after 10 days propagation (D10) it changed in favor of *Saccharomyces* (3.3 ± 0.4%), *Claviceps* and *Blumeria*, which increased from 2.3 ± 0.3% and 0.6 ± 0.04% at D0 to 3.1 ± 0.2% and 0.8 ± 0.07% at D10, respectively. *Alternaria* (21.6 ± 0.3%) and *Microdochium* (15.9 ± 0.4%) decreased slightly at the end of 10 days of sourdough propagation (Fig. S3B and Fig. 3D).

3.4. Biochemical features

The biochemical features (pH, TTA, organic acids, FQ, and carbohydrates) of *Tritordeum* dough and sourdoughs were analyzed as a function of propagation time (Table 1). During the first three days of sourdough preparation and propagation (from D0 to D3), the biochemical features exhibited minimal changes. After 4 days (D4), the sourdough can be considered in the transition phase, followed by its stabilization from day 5 onwards (Table 1). The pH-value in the dough prior to the first fermentation (D0) was 5.83 ± 0.04, then it significantly ($P < 0.05$) decreased after 4 days of propagation and it stabilized from day 5 (D5) onwards. After 10 days of refreshment, the mature sourdoughs had pH values of 4.31 ± 0.03 (Table 1). As indicated by pH values, the titratable acidity (TTA) significantly ($P < 0.05$) increased in parallel throughout the time (Table 1). Sucrose, fructose, and glucose were the predominant carbohydrates in dough before the first fermentation (9.80 ± 0.69, 6.45 ± 1.39 and 4.24 ± 0.24 mM, respectively). Sucrose significantly ($P < 0.05$) decreased over time and almost disappeared after 10 days of propagation (0.02 ± 0.02 mM). Fructose and glucose significantly ($P < 0.05$) increased during the following three days, then they progressively decreased from the fourth day (D4) onwards, but they were still detectable after 10 days fermentation (Table 1). Concomitantly with fructose consumption, mannitol was released from the fourth day (3.16 ± 0.28 mM) onwards of propagation. The main microbial metabolites were lactic and acetic acids (Table 1). During the first 3 days, a slight but significant ($P < 0.05$) increase of both organic acids was found, which markedly increased at each further timepoint until the end of the propagation. Fermentation quotient (FQ), defined as the ratio of molar concentrations of lactic and acetic acids, decreased throughout the sourdough propagation, falling in the optimal range from 6 to 10 days (range 4.26 ± 0.32–4.08 ± 0.15).

The influence of prevalent sourdough microbiota on the biochemical characteristics was shown through the Pearson correlation matrix (Fig. 4), indicating clear negative or positive correlations between microbial taxa and biochemical parameters. *Lactobacillus* and *Claviceps* showed positive correlations with lactic acid, acetic acid, TTA and mannitol, and negative correlations with pH, sucrose, FQ, glucose and fructose. Conversely, *Erwinia*, *Pantoea*, *Microdochium*, *Kosakonia* and *Enterobacteriaceae* displayed opposite correlations with the biochemical features. The individual correlation analysis of each biochemical parameter is reported in the Supplementary material (Fig. S4A–I).

3.5. Nutritional characterization

Prior to the first fermentation (D0), dough contained 10 ± 1.32 mg gallic acid eq. 100 g^{-1} d.m. of total phenolic concentration, which progressively ($P < 0.05$) increased during the propagation, reaching the highest values at the end of the propagation (50 ± 2.52 mg gallic acid eq. 100 g^{-1} d.m.) (Fig. 5). Similarly, the antioxidant activity, as determined by the radical scavenging activity on DPPH radical, progressively increased from the first to the tenth day of propagation (Fig. 5).

Dietary fibers (DF) (total, soluble, and insoluble), total free amino acids (TFAA), antinutritional factors (phytic acid and raffinose) were determined in the dough before the first fermentation (D0), and in sourdough after 5 and 10 days of propagation (Table 2).

Dough before the first fermentation had a content of total DF of 5.59 ± 1.16% dry weight, consisting of insoluble (2.46 ± 0.64% dry weight) and soluble (3.13 ± 0.55% dry weight) DF (Table 2). Sourdough propagation after 5 and 10 days, favored a significant ($P < 0.05$) percentage increase of total (38 and 50%), soluble (24 and 14%) and insoluble (50 and 67%) DF. Simultaneously, mature sourdough (D5 onwards) influenced the TFAA concentration, which was shown to be significantly ($P < 0.05$) increased from 0.65 ± 0.05 mg/kg (day 0) to 1.01 ± 0.09 mg/kg (day 10) during successive sourdough propagations. The antinutritional

Closest relative (% identity)	Genbank Accession No.	D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Weissella confusa (100)	NR_113258.1	◕	◕	◕	◕	◕	◑	◔	◔	◑	◔	◔
Weissella confusa/W. cibaria (99)	NR_113258.1/ NR_036924.1	◑	◑	◔	◑	◑	◔	◔	◴	◴	◴	◴
Weissella paramesenteroides (98)	NR_104568.1	◴	◴	◴	◴	◴	◴	◴	○	○	○	○
Lactiplantibacillus plantarum (99)	NR_104573.1	○	○	○	○	○	◑	◑	◑	◑	◑	◑
Latilactobacillus curvatus (99)	NR_042437.1	○	○	○	○	○	◴	◴	◴	◴	◴	◴
Leuconostoc mesenteroides (99)	NR_074957.1	○	○	○	○	○	◴	◴	◴	◴	◴	◴
Saccharomyces cerevisiae (99)	MN559526.1	●	●	●	●	◕	◕	◕	◕	◕	◕	●
Clavispora lusitaniae (100)	KY106929.1	○	○	○	○	◴	◴	◴	◴	◴	◴	◴
Pichia kudriavzevii (99)	KX237674.1	○	○	○	○	○	○	○	◴	◴	◴	○

Fig. 2. Species of lactic acid bacteria and yeasts identified through the culture-dependent method during the preparation and propagation of *Tritordeum* sourdough. Samples were taken after dough mixing and before fermentation (D0) and after 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 (D1–D10) days of sourdough propagation. The individual pie charts in each cell represent the percentage of identified microorganism at the respective day of propagation. 0% (○), 1–5% (◴), 6–15% (◔), 20–30% (◑), 50% (◑), 51–80% (◕), 81–90% (◕), 100% (●).

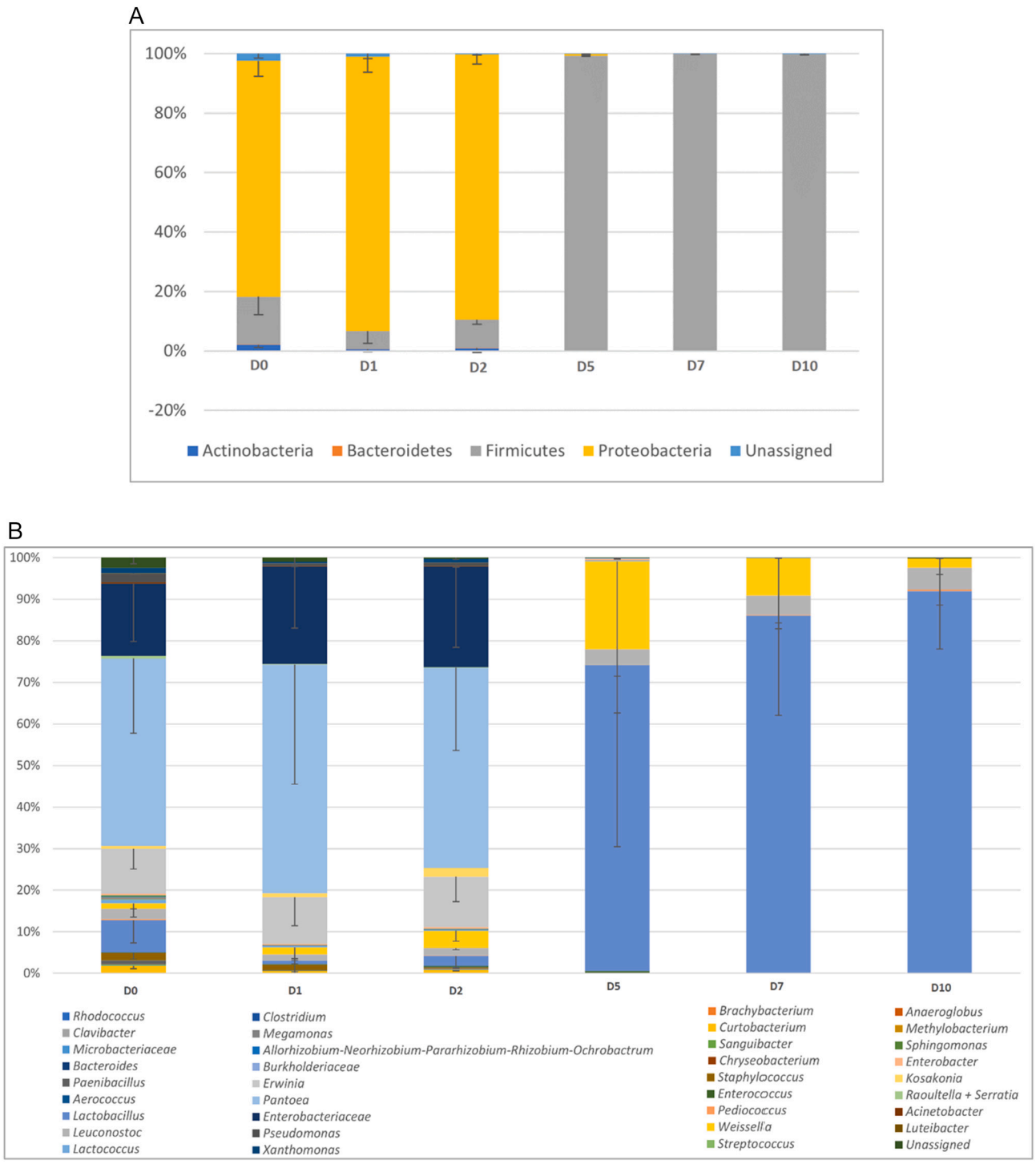

Fig. 3. Relative abundances (%) of bacteria (A–B) and yeasts (C–D) at phylum and genus level from *Tritordeum* doughs prior (D0) and after the first fermentation (D1), and after 2 (D2), 5 (D5), 7 (D7) and 10 (D10) days propagation. The values are represented as Mean abundances with error bars indicating the standard deviation between the replicates ($n = 3$).

factors, determined as phytic acid and raffinose concentrations, were diminished to 0.04 ± 0.03 and 0.14 ± 0.07 g/100 g, respectively, after 10 days of continuous sourdough propagation (Table 2).

3.6. Textural profile analysis of bread

Sourdough after 10 days of propagation (D10) was used as leavening agent for bread making. As expected, the use of sourdough significantly ($P < 0.05$) affected the pH value and the texture profile of bread. The addition of sourdough lowered the pH of *Tritordeum* bread (4.9 ± 0.16 units) as compared to *Tritordeum* bread prepared with commercial baker's yeast (5.3 ± 0.21 units). Moreover, when compared to baker's yeast bread, the cohesiveness and resilience of the *Tritordeum* sourdough bread were significantly ($P < 0.05$) higher (4179 ± 978.9 and 2071 ± 893.9 vs 2680 ± 252.7 and 554 ± 125.4, cohesiveness and resilience, respectively for *Tritordeum* sourdough and for *Tritordeum* baker's yeast

Fig. 3. (*continued*).

bread, $P < 0.05$) The same trend was found for the firmness measured for both crust and crumb (2273 ± 862.5 N vs 1620 ± 538.9 and 1450 ± 579.5 N vs 868.67 ± 40.0, respectively for *Tritordeum* sourdough and for *Tritordeum* baker's yeast bread).

4. Discussion

The cultivation of *Tritordeum* as an alternative cereal for making health-promoting foods is increasing at the European level. Its resilience to harsh environments (drought, salinity, and extreme temperature) (Villegas et al., 2010), combined with its nutritional profile (Visioli et al., 2020) make it a cereal with high potential for sustainable

Table 1
Biochemical characterization during the preparation and propagation of *Tritordeum* sourdough up to 10 days. D0 represents dough prior to fermentation and before becoming sourdough. D1–D10 represent days of sourdough propagation. Data are the means of three independent experiments ± standard deviations ($n = 3$) analyzed in triplicates. Statistical analysis was performed by one-way ANOVA. Values in the same column with different superscript letters differ significantly ($P < 0.05$).

Day of sourdough propagation	pH	Total titratable acidity (TTA)* (ml)	Lactic acid (mM)	Acetic acid (mM)	Fermentation Quotient (FQ)**	Glucose (mM)	Fructose (mM)	Sucrose (mM)	Mannitol (mM)
D0	5.83 ± 0.04^{b}	1.96 ± 0.21^{g}	15.58 ± 0.35^{g}	1.16 ± 0.10^{g}	13.54 ± 1.29^{a}	4.24 ± 0.24^{e}	6.45 ± 1.39^{d}	9.80 ± 0.69^{b}	n.d.
D1	5.89 ± 0.04^{a}	2.07 ± 0.06^{g}	14.65 ± 0.21^{h}	1.76 ± 0.09^{f}	8.36 ± 0.45^{b}	13.69 ± 2.12^{a}	10.71 ± 0.87^{a}	10.70 ± 1.14^{a}	n.d.
D2	5.85 ± 0.04^{ab}	2.10 ± 0.10^{g}	15.37 ± 0.39^{g}	1.77 ± 0.17^{f}	8.74 ± 0.98^{b}	14.66 ± 1.45^{a}	10.16 ± 0.55^{b}	7.35 ± 1.79^{b}	n.d.
D3	5.79 ± 0.02^{b}	2.29 ± 0.08^{f}	26.52 ± 1.25^{f}	2.99 ± 0.40^{e}	8.99 ± 1.45^{b}	10.33 ± 1.90^{b}	10.12 ± 1.32^{ab}	5.85 ± 1.29^{c}	n.d.
D4	5.19 ± 0.04^{c}	3.00 ± 0.10^{e}	104.75 ± 9.37^{e}	17.74 ± 1.64^{d}	5.93 ± 0.66^{c}	7.48 ± 1.24^{c}	12.97 ± 1.44^{a}	0.60 ± 0.46^{d}	3.16 ± 0.28^{d}
D5	4.52 ± 0.04^{d}	5.18 ± 0.08^{d}	180.49 ± 8.76^{cd}	35.66 ± 1.96^{c}	5.08 ± 0.42^{d}	6.53 ± 1.13^{c}	8.41 ± 0.97^{c}	0.22 ± 0.08^{d}	7.81 ± 2.55^{c}
D6	4.53 ± 0.03^{d}	5.18 ± 0.03^{d}	176.64 ± 4.99^{d}	41.66 ± 3.45^{b}	4.26 ± 0.32^{e}	5.60 ± 0.78^{d}	7.09 ± 0.23^{c}	0.02 ± 0.01^{e}	8.42 ± 0.98^{c}
D7	4.39 ± 0.05^{e}	5.23 ± 0.15^{d}	185.53 ± 7.44^{c}	43.18 ± 4.07^{b}	4.33 ± 0.51^{e}	5.39 ± 0.64^{cd}	5.92 ± 0.61^{d}	0.04 ± 0.022^{e}	11.64 ± 1.53^{b}
D8	4.35 ± 0.05^{ef}	5.80 ± 0.10^{c}	201.90 ± 5.35^{b}	54.56 ± 3.19^{a}	3.71 ± 0.14^{f}	5.28 ± 0.81^{cd}	4.52 ± 0.91^{e}	0.02 ± 0.005^{e}	13.11 ± 2.05^{a}
D9	4.32 ± 0.04^{ef}	6.07 ± 0.06^{b}	226.56 ± 4.83^{a}	54.03 ± 3.91^{a}	4.20 ± 0.22^{e}	6.34 ± 0.76^{c}	5.38 ± 1.07^{d}	0.03 ± 0.02^{e}	15.35 ± 2.32^{a}
D10	4.31 ± 0.03^{ef}	6.23 ± 0.06^{a}	229.83 ± 6.03^{a}	56.33 ± 2.71^{a}	4.08 ± 0.15^{e}	6.30 ± 0.78^{c}	5.06 ± 0.75^{de}	0.02 ± 0.02^{e}	15.66 ± 1.29^{a}

* Total titratable acidity (TTA) is expressed as the volume of 0.1 N NaOH/10 g of dough.
** Fermentation Quotient (FQ) is the ratio of molar concentrations of lactic and acetic acids.

agriculture. Although previous studies have shown that *Tritordeum* is more suitable for bread making than for pasta making (Martín et al., 1999), to date it appears not to have been evaluated for making sourdough bread. In this perspective, the ecological dynamic of *Tritordeum* flour fermentation may provide useful information for its exploitation for leavened baked goods production. In this study, we evaluated the microbial community, biochemical and nutritional properties of *Tritordeum* sourdough-type I fermentation during 10 days of backslopping procedure under controlled laboratory conditions, which markedly restricted environmental contamination. The sourdough propagation conditions chosen in this study were similar to traditional protocols previously used for cereal sourdough fermentation (Ercolini et al., 2013; Minervini et al., 2012; Rizzello et al., 2014). Dough (flour and water; day 0) was characterized for a low cell density of LAB (ca. 3.5 log CFU g^{-1}) and yeasts (ca. 2 log CFU g^{-1}). After five days of propagation, LAB dominated the sourdough, reaching a cell density of ca. 8.5 log CFU g^{-1} and remained at this level until the end of the 10-days propagation, indicating a stable bacterial community, as commonly observed for cereal sourdoughs (De Vuyst and Neysens, 2005; Ercolini et al., 2013). The yeast initial cell density remained constant until day 4, but thereafter it increased to 5.0 ± 0.29 log CFU g^{-1}. A similar trend was previously observed in spontaneous fermentation of legumes and mixed cereal flours, where yeast counts were either not detectable after the second backslopping step (Coda et al., 2017) or were only detected after 5 up to 10 days of propagations (Rizzello et al., 2014). Spontaneous fermentations performed in the laboratory under controlled conditions, may be less prone to yeast contamination compared to the bakeries, where baker's yeast is usually present (De Angelis et al., 2019). Further, the trophic relationships between LAB/yeasts during sourdough fermentation, can diversify the utilization of available carbon sources and consequently influence their progressive growth, competitiveness and/or adaptability when they are coexisting (Gobbetti, 1998). As shown by plate count, after the fourth backslopping, *Enterobacteriaceae* decreased and were no longer detected on day 10 of sourdough propagation. Complementary biochemical features suggested that sourdoughs achieved maturity during 5 to 7 days of propagation. Maturity refers to a sourdough that has reached constant technological properties (e.g., acidification, leavening capacity). At this time, the ratio between LAB and yeasts stabilized to ca. 1000:1 (Comasio et al., 2020), and the acidification became constant (pH ca. 4.3). The leavening power was low for all sourdoughs analyzed throughout the propagations due to the controlled fermentation conditions as well as the barley component in *Tritordeum* flour. In fact, it has been previously demonstrated that the high fiber content in barley inhibits the formation of gas cells leading to decrease in leavening power during fermentation (Izydorczyk and Dexter, 2008).

The analysis of the 16S rRNA gene and ITS sequences showed that the microbial diversity decreased during propagation, as previously reported for Type I sourdoughs (Ercolini et al., 2013; Rizzello et al., 2015). Dough was dominated by *Pantoea*, *Enterobacteriaceae*, *Lactiplantibacillus* and *Erwinia*, which persisted during the first fermentation and until 2 days of propagation (Fig. 3B). *Pantoea*, *Erwinia* and *Enterobacteriaceae* are commonly detected in flours as contaminants, but upon fermentation and subsequent propagations progressively decrease in abundance (Ercolini et al., 2013). In fact, the increase of *Lactiplantibacillus* (73.6 ± 43.7%) after 5 days propagation corresponded to a massive decrease of *Pantoea*, *Enterobacteriaceae* and *Erwinia*, which disappeared after 7- and 10-days propagation. This suggested the leading role of *Lactiplantibacillus* and the low competitiveness or inhibition of the other genera throughout propagations. *Weissella* was detected in all samples, showing the highest relative abundance after 5 days propagation and a significant decrease after 7- and 10-days propagation. The presence of the above-mentioned genera is consistent with the bacterial succession usually observed in spontaneous cereal fermentations, which is characterized by *Enterobacteriaceae*, *Enterococcus*, *Leuconostoc*, *Pediococcus* and/or *Weissella* species until highly competitive acid-tolerant lactobacilli are selected through the backslopping procedure and dominate in the sourdough (Dinardo et al., 2019). The fungal community was quite stable during propagation, with *Alternaria* and *Microdochium* dominating dough and sourdough samples (Fig. 3D). Focusing on the yeast genera, an increase of *Saccharomyces* was observed only after 10 days propagation. The next-generation sequencing results were confirmed by culture-dependent methods. Indeed, LAB dominated throughout the propagation, yeasts increased by ca. 2 logarithmic units from day 5 onwards, while *Enterobacteriaceae* progressively decreased and disappeared by day 10 of sourdough propagation, suggesting that is not

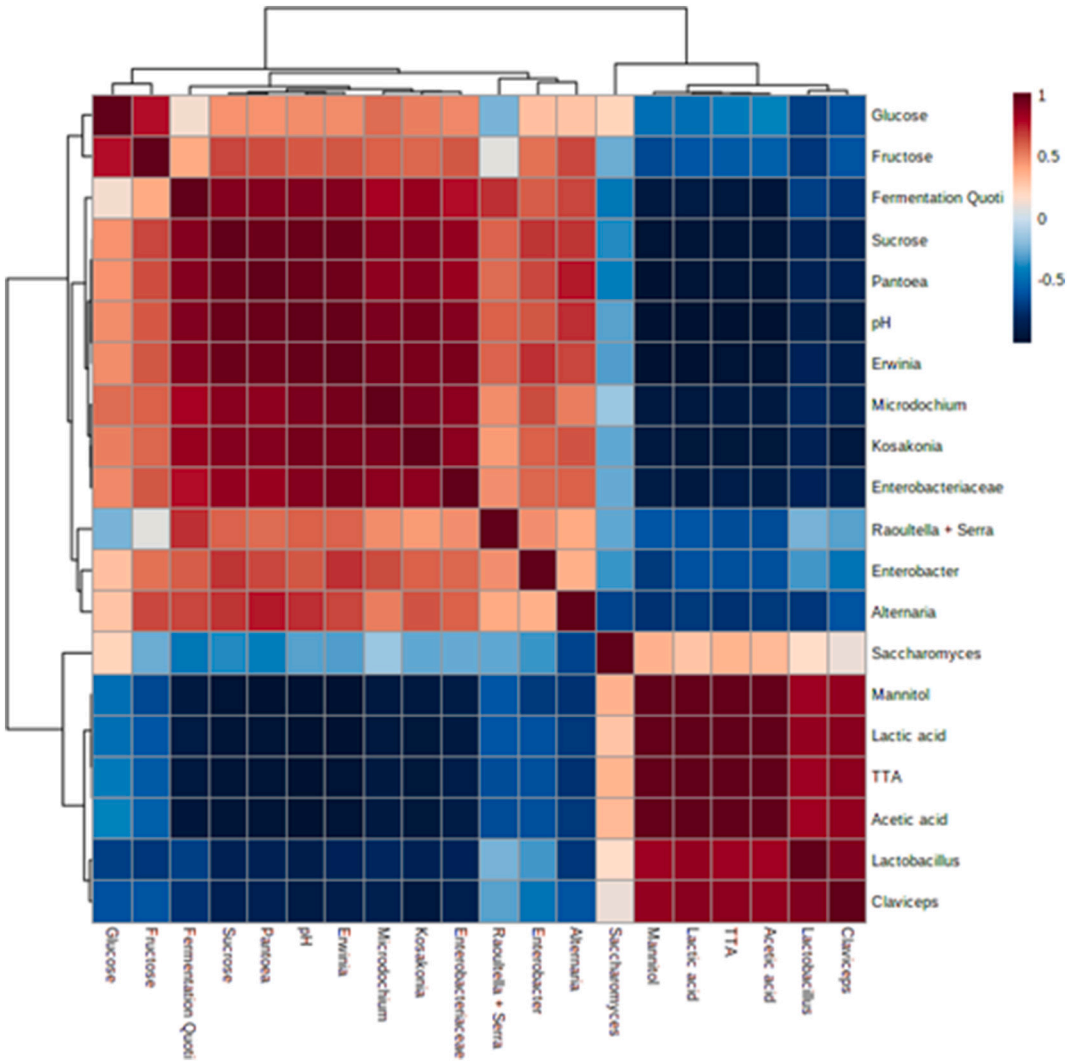

Fig. 4. Pearson's correlation matrix between the dominant sourdough microbiota estimated by 16S rRNA gene sequencing and the biochemical characteristics as observed throughout the *Tritordeum* sourdough propagation. Strong positive correlations ($P < 0.05$, $r > 0.5$) are given with red deep color, while strong negative correlations ($P < 0.05$, $r < 0.5$) with deep blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

able to survive to a long-term acidification process. Among the isolates, amplicon sequencing of 16S rRNA gene identified *W. confusa* as the prevalent LAB species throughout the sourdough preparation and propagation, while L. *plantarum*, *L. curvatus* and *Leuc. mesenteroides* dominated the mature sourdough from day 5 onwards. These species have previously identified as typical cereal sourdoughs microbiota members (De Vuyst et al., 2014; Minervini et al., 2012). *S. cerevisiae* was the only yeast isolated from the first to the last day of propagation, while *C. lusitaniae* and *P. kudriavzevii* were only identified in the mature *Tritordeum* sourdough.

Notable changes in biochemical and nutritional features of sourdough were found from the transition point (day 4) to the stabilization point of mature sourdough (day 5–7) until the end of propagations (day 10). The technological characteristics followed the bacterial succession over time. Production of lactic and acetic acid was minimal until the transition point (day 4), while they significantly increased after stabilization and until end of propagations. The FQ, defined as the ratio of molar concentrations of lactic and acetic acids, decreased with each propagation, showing optimal values from day 5 to day 10 (ca. 3.5–4.0). FQ has a considerable effect on the flavor of baked goods as long as for the shelf life of breads, considering values ranging between 1.5 and 4 (De Luca et al., 2021). Microbial activity and composition mostly influences the variation in the FQ, primarily associated with the ratio between homo- and heterofermentative LAB. Yeast activities may also play a role liberating fructose, which in turn will induce acetic acid production by LAB and decrease the FQ value (Corsetti, 2013). This was evident from culture independent methods, with a dominance of heterofermentative LAB (*L. plantarum*, *L. curvatus* and *Leuc. mesenteroides*) observed after day 5.

The traditional fermentation process led to the decrease of the concentration of glucose, fructose, and sucrose. Decrease of glucose and fructose between 1- and 10- days refreshment was >50%, while the consumption of sucrose ranged between 90 and 99% after 5- and 10-days propagation, respectively. These sugars are converted into lactic acid and/or acetic acid, ethanol and carbon dioxide by homo- and/or heterofermentative LAB. Heterofermentative LAB, which dominated the mature sourdough, also produced mannitol, most likely using fructose as an electron acceptor (Wisselink et al., 2002). The acidic environment of sourdough is likely to have inhibited the growth of *Erwinia*, *Pantoea*, *Microdochium*, *Kosakonia* and *Enterobacteriaceae* (Ercolini et al., 2013), as supported by the positive correlation with pH and a negative correlation with TTA ($P < 0.05$). Conversely, *Lactobacillus* showed positive correlations with lactic acid, acetic acid, TTA and mannitol, and negative correlations with pH, sucrose, FQ, glucose and fructose ($P < 0.05$).

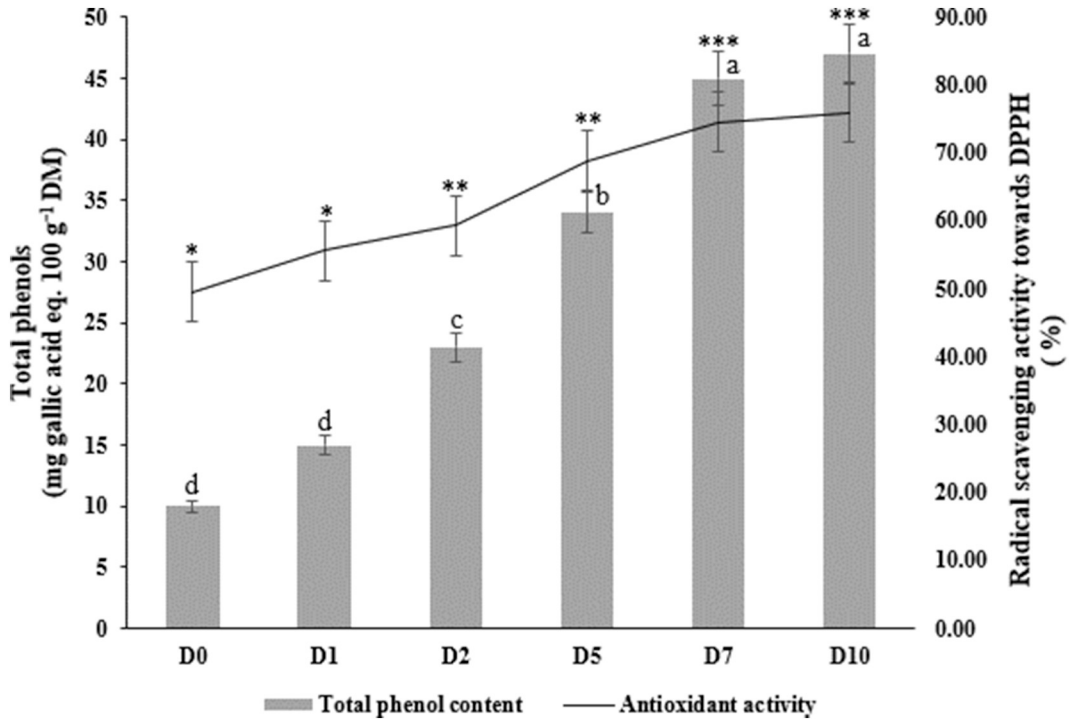

Fig. 5. Total phenol content (mg gallic acid eq. 100 g^{-1} D.M) (grey bars) and DPPH radical scavenging activity (%) (line) of methanol soluble extracts (MSE) from *Tritordeum* dough prior (D0) and after the first fermentation (D1), and sourdoughs after 2 (D2), 5 (D5), 7 (D7) and 10 (D10) days of propagation. Data are the means ± standard deviation of three independent experiments analyzed in triplicates. Total phenol content is given in the principal y-axis (left), while DPPH radical scavenging activity in the secondary y-axis (right).

Table 2
Nutritional characterization during the preparation and propagation of *Tritordeum* sourdough up to 10 days, in terms of dietary fiber content and total free amino acids, and antinutritional factors (phytic acid and raffinose). Data are the means of three independent experiments ± standard deviations ($n = 3$) analyzed in triplicates. Statistical analysis was performed by one-way ANOVA. Values in the same column with different superscript letters differ significantly ($P < 0.05$).

Day of sourdough propagation	Dietary Fiber (DF) content (%)			Total free amino acids (mg/kg)	Phytic acid (g/100 g)	Raffinose (g/100 g)
	Soluble DF	Insoluble SF	Total DF			
Day 0	3.13 ± 0.55^{b}	2.46 ± 0.64^{c}	5.59 ± 1.16^{c}	0.65 ± 0.05^{c}	0.52 ± 0.06^{a}	0.84 ± 0.11^{a}
Day 5	4.13 ± 0.47^{a}	4.97 ± 0.51^{b}	9.1 ± 0.69^{b}	0.82 ± 0.07^{b}	0.21 ± 0.03^{b}	0.16 ± 0.05^{b}
Day 10	3.58 ± 0.48^{a}	7.59 ± 0.79^{a}	11.17 ± 1.23^{a}	1.01 ± 0.09^{a}	0.04 ± 0.03^{c}	0.14 ± 0.07^{b}

Lactobacillus species are highly adaptable to the acidic environment, dehydration, and nutrient depletion during propagations, which enable their natural selection and final dominance in the sourdough ecosystem (Zhang et al., 2019).

An increase of total phenols and antioxidant activity was detected during propagations, with the highest values in the mature sourdoughs. This result is consistent with the dominance of LAB from day 5 onwards, which have been recognized for their esterase activity and ability to hydrolyze complex phenolic compounds and their glycosylated forms into the corresponding phenolic acids (Nionelli et al., 2014). *Tritordeum* has been reported as a rich source of dietary fibers attributed to its barley component (Vaquero et al., 2018), and the brewer's spent grain (BSG) derived from *Tritordeum* has been used to increase the nutritional content of durum pasta (Nocente et al., 2021). The total dietary fiber content increased during propagations, with IDF content higher than SDF. This effect could be attributed to durum wheat constituent of *Tritordeum* (Saa et al., 2018), and to biological acidification contributed by sourdough LAB, which is one of the main factors that decreases starch hydrolysis rate (Curiel et al., 2015). Fermentation with LAB has been largely reported to be an efficient method for improving the protein bioavailability and overcoming the presence of antinutritional factors (Gänzle, 2020). Here, the concentration of total free amino acids was increased in *Tritordeum* as a result of fermentation, which is likely related to an intense proteolytic activity by endogenous and microbial enzymes (Gänzle, 2020; Rizzello et al., 2019). The use of sourdoughs also led to a reduction of antinutritional factors (phytic acid and raffinose), known for limiting the digestibility (Rizzello et al., 2019).

Tritordeum sourdough breads were prepared and compared with commercial baker's yeast *Tritordeum* breads. All samples exhibited yellowish color of the crumb. Other parameters, such as crust and crumb firmness, cohesiveness (crumb integrity) and resilience (crumb recovery), were superior in *Tritordeum* sourdough bread. The production of LAB metabolites, such as mannitol and acetic acid have been previously shown to improve the bread rheology and its shelf life (Korakli et al., 2001).

5. Conclusion

An exhaustive variety of raw materials (cereals, pseudo-cereals, legumes, by-products etc.) have been exploited to date for sourdough fermentation. In this context, the sourdough fermentation can add functional value to a sustainable and novel cereal, *Tritordeum*. The type I *Tritordeum* sourdough possessed characteristic biochemical features influenced by the sourdough microbiota and an increased nutritional potential, due to the inherent combination of wheat and barley in this golden cereal. The bread produced with *Tritordeum* sourdough had

firmer and more defined texture compared to the baker's yeast bread. Overall, our findings demonstrate the improved nutritional value of *Tritordeum* cereal by sourdough fermentation to be employed successfully in the manufacture of baked goods addressed to consumers oriented to prefer healthy and highly nutritious bread, such sourdough-based.

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CRediT authorship contribution statement

Kashika Arora: Conceptualization, Investigation, Data curation and Writing – original draft preparation. **Ilaria Carafa**: Data curation and Writing – reviewing and editing. **Francesca Fava**: Supervision and Writing – reviewing and editing. **Kieran M. Tuohy**: Supervision and Writing – reviewing and editing. **Olga Nikoloudaki**: Writing – reviewing and editing. **Marco Gobbetti**: Conceptualization and Supervision. **Raffaella Di Cagno**: Conceptualization, Supervision, Project administration and Writing – reviewing and editing.

Declaration of competing interest

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

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