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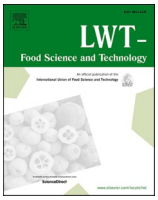

Microbial ecology, biochemical and nutritional features in sprouted composite type I sourdough made of wheat and blend flours

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A B S T R A C T

Germination is a biotechnological process helpful in obtaining new plants from grains. We tested the impact of processing condition (sprouting and dough yield) and flour composition on type I sourdoughs biochemical and nutritional parameters we here used four flour matrices and precisely: i) refined commercial wheat flour, ii) sprouted and iii) non-sprouted whole wheat flour and iv) a blend composed of sprouted whole wheat and sprouted lentils. We here compared a set of 24 samples based on different group stratifications including three different sampling times (starting, after 24h and at the 10th refreshment). Moreover, we inspected the microbiota and its relative taxa abundance by 16S rRNA target sequencing and qPCR absolute quantification. Our result highlighted how the sprouted process together with the dough yield influenced key substrate like raffinose and lead to a change of taxa composition as evinced for the increased relative abundances of *Pediococcus* genus. Other key taxa in the microbiota were shaped by tested conditions and the alpha and beta diversity evidenced how matrix impact the intergroup clustering. The presented results shed new light on the increased properties and the related health promoting effects of type I sourdough obtained with the sprouting process.

1. Introduction

Food producers are interested in healthy and natural ingredients that can improve functional and nutritional qualities. In recent past years, much evidence has been gained in favour of sprouted grains that grant beneficial effects on human health and wellness. This proven strength boosted sprouted product launch on the global market (Žilić et al., 2016). As a biotechnological process, germination has become helpful in transforming grain into new plants, leading to cereal and legume's nutritional value improvement, driven by physiological and biochemical changes. As a consequence of the germination process, the final sprouted flour compositional profiles results to be enriched in terms of macro- and micro-nutrients as well as in terms of bioactive compound (Benincasa et al., 2019; Singh et al., 2015). According to our previous study (Perri et al., 2020), wheat and lentil sprouting leads to changes in lactic acid bacteria (LAB) and yeasts cell density. Concomitantly, it was also observed how phenolic compounds, soluble fibers and sugars resulted increased as the direct effect of flours microbiota. In this study, sprouted grains acquired a valuable potential as ingredients to be used in functional food production. Moreover, the evidence that sourdough fermentation can enhance sprouted food health promoting features (Gobbetti et al., 2020), pushes towards the need for newly and alternative flours obtained from cereals, pseudo-cereals, legumes and food by-products (Arora et al., 2021; Gobbetti et al., 2020; Perri et al., 2021b; Rizzello et al., 2014). In fact, during sourdough fermentation the dominant microbial community, responsible for acid production, and that practically cause the dough leavening, mainly consists of LAB and yeasts (Bessmeltseva et al., 2014; De Vuyst & Neysens, 2005; Gobbetti et al., 1995). Lactic acid fermentation has a crucial role in the bioprocessing of sprouted grain, acting on the decrease activity of antinutritional factors (Perri et al., 2021b; Perri et al., 2021a). To date, despite the extensive characterization of sprouted grains (Dziki et al., 2015; Yang and B. Ooraikul, 2001), the evolution of the relative bacterial consortia and the nutritional features in spontaneously fermented sourdough has not been described yet. The microbial ecology dynamics featuring wheat and legume sourdough preparation have been characterized and interpreted by meaning of a high-throughput sequencing approach (Ercolini, 2013; Galli et al., 2020). Noteworthy, the stability of the sourdough microbiota, together with the mutual interconnection existing between the commensal taxa, depends on several ecological drivers, accounting both for technological and environmental parameters (De Vuyst et al., 2009; Minervini et al., 2014). Furthermore, the

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metabolic adaptability to stress conditions, the nutritional interactions among microorganisms, and the intrinsic dominance or disadvantage of microorganisms impact the mature sourdough stability (Minervini et al., 2014).

In this study, sprouted and non-sprouted flours were used in traditional type I sourdough biotechnology, based on the backslopping procedure and two different dough yield conditions (firm 160 and liquid 280). Our results investigate changes in biochemical and nutritional variables, as well in the microbiota composition and abundance, inspected both by 16S rRNA and qPCR approaches. All the gathered variables were here analysed based on the dough yield condition (firm or liquid), sprouting process and time of propagation in four different theses.

2. Materials and methods

2.1. Flour

Two different grains were basically considered in this study: a first made of a cereal (wheat (W), *Triticum durum* var. Simeto), and a second composed of a legume (lentil (L), *Lens culinaris*). Cereals and legumes were purchased by Caporal Grani S. a.s. (Gravina di Puglia, Bari, Italy). Grains were germinated according to (Montemurro et al., 2019) with some modifications described by Perri et al., (2021a) (Supplementary Fig. S1). Sprouted and non-sprouted sample grains were milled separately into smaller particle sizes (<500 μm) by using a laboratory mill (Ika-Werke M20 GMBH, and Co. KG, Staufen, Germania), without removing the rootlets. Four different flours were used for sourdough preparation as described in the following paragraph: i) refined commercial wheat flour (RW); ii) whole non-sprouted wheat (NSW); iii) whole sprouted wheat (SW); iiii) composite flour containing 80% of whole sprouted wheat (SW) and 20% of whole sprouted lentil (SWSL). (Rizzello et al., 2014).

Grain gross compositions were the following: i) for the refined wheat (RW), moisture = 15.6 ± 1.0%, protein (N × 5.7) = 10 ± 0.5% of dry matter (d.m.); total carbohydrates = 68 ± 2.5%, of d.m.; dietary fiber = 1.5 ± 0.7% of d.m., and fat = 1.0 ± 0.11 of d.m.; ii) for wheat (W), moisture = 13.6 ± 1.0%, protein (N × 5.7) = 17 ± 0.5% of dry matter (d.m.); total carbohydrates = 66.5 ± 2.5%, of d.m.; dietary fiber = 3.2 ± 0.7% of d.m., and fat = 1.9 ± 0.11 of d.m.; iii) for lentil (L), moisture, 11.1 ± 0.5%, protein (N × 5.7), 30.0 ± 1.0% of dry matter (d.m.); total carbohydrates, 50.3 ± 1.5%, of d.m.; dietary fiber, 23.2 ± 0.8% of d.m., and fat 0.70 ± 0.05 of d.m. Three different batches, one for each flour type, were pooled and used as raw materials to formulate the sourdoughs.

2.2. Dough preparation and sourdough propagation

Preparation of dough and propagation of sourdough were performed by following traditional protocols (Di Cagno et al., 2014; Minervini et al., 2012), without the addition of starter cultures or baker's yeast. Sourdoughs were obtained starting from four type of flours (raw wheat (RW), non-sprouted wheat (NSW), sprouted wheat (SW), sprouted wheat/sprouted lentils (SWSL)), both in firm (dough yield 160, DY160) and liquid (dough yield 280, DY280) conditions (Table 1 and Supplementary Fig. S1).

Firm dough preparation was obtained as follow: 125 g of flour and 65 mL of tap water were used to produce 200 g of dough. For liquid dough, flours (71.4 g) were mixed with tap water (128.6 mL) (Di Cagno et al., 2014) and the liquid mixtures were kept homogeneous through a stirring step (100 rpm). The first fermentation step (firm and liquid dough) was carried out at 30 °C for 24 h. Subsequently, daily backsloppings (refreshments, R) were repeated for 10 days (from R1 to R10), by mixing 15% of the previously fermented dough with a new mixture of flour and water (reaching final dough yield 160 or 280) then incubated at 30 °C for 8 h. At the end of each refreshment step, sourdoughs were cooled down at 4 °C. Samples were collected after 0h (D0, dough prior to fermentation and before becoming sourdough), 24h (D24, after the first 24h of fermentation), and at the 10th refreshment (R10). All cooled sourdough samples were analysed after 2 h after collection. Considering four different sourdough types at three different times and in two DY conditions, we analysed a total of 24 samples as reported in Table 1.

Table 1
Sample cohort and associated metadata. Sample matrices were: i) refined commercial wheat flour (RW); ii) whole non-sprouted wheat (NSW); iii) whole sprouted wheat (SW); iiii) a blend flour composed of 80% of whole sprouted wheat and 20% of whole sprouted lentil (SWSL). Sample metadata included quantity of flour and water, sprouted or non-sprouted condition; dough yield (160 = firm or 280 = liquid); backslopping time (D0 = prior fermentation, after 24h of fermentation = D24, after the 10th refreshment = R10. The amount of each ingredient refers to 200 g of dough.

Sample _ID	Sprouting	Flour (g)[c]	Water (g)[c]	Dough Yield	Backslopping
RW_F_D0	Non_Sprouted	125	65	160	0h
RW_F_D24	Non_Sprouted	125	65	160	24h
RW_F_R10	Non_Sprouted	125	65	160	10 days
RW_L_D0	Non_Sprouted	71.4	128.6	280	0h
RW_L_D24	Non_Sprouted	71.4	128.6	280	24h
RW_L_R10	Non_Sprouted	71.4	128.6	280	10 days
NSW_F_D0	Non_Sprouted	125	65	160	0h
NSW_F_D24	Non_Sprouted	125	65	160	24h
NSW_F_R10	Non_Sprouted	125	65	160	10 days
NSW_L_D0	Non_Sprouted	71.4	128.6	280	0h
NSW_L_D24	Non_Sprouted	71.4	128.6	280	24h
NSW_L_R10	Non_Sprouted	71.4	128.6	280	10 days
SW_F_D0	Sprouted	125	65	160	0h
SW_F_D24	Sprouted	125	65	160	24h
SW_F_R10	Sprouted	125	65	160	10 days
SW_L_D0	Sprouted	71.4	128.6	280	0h
SW_L_D24	Sprouted	71.4	128.6	280	24h
SW_L_R10	Sprouted	71.4	128.6	280	10 days
SWSL_F_D0	Sprouted	100 SW, 25 SL	65	160	0h
SWSL_F_D24	Sprouted	100 SW, 25 SL	65	160	24h
SWSL_F_R10	Sprouted	100 SW, 25 SL	65	160	10 days
SWSL_L_D0	Sprouted	57.12 SW, 14.28 SL	128.6	280	0h
SWSL_L_D24	Sprouted	57.12 SW, 14.28 SL	128.6	280	24h
SWSL_L_R10	Sprouted	57.12 SW, 14.28 SL	128.6	280	10 days

2.3. Enumeration of cultivable microorganisms

Ten grams of D0, D24, and R10 samples were homogenized in 90 ml of sterile NaCl solution (0.9% w/v) in a stomacher bag (Bag Mixer, Interscience International, Roubaix, France) for 3 min, as reported in (Perri et al., 2020). A tenfold dilution series of this suspension was made and used for the enumeration of cultivable microorganisms. Total mesophilic aerobic microorganisms were enumerated on Plate Count agar (PCA) (Oxoid, Basingstoke, Hampshire, UK), after incubation at 30 °C for 48h. The presumptive lactic acid bacteria were assessed on three different media and precisely i) modified de Man-Rogosa-Sharpe agar medium (mMRS) composed of 5 g/L maltose and 5 g/L fresh yeast extract at pH 5.6 (M.R.S. agar, Oxoid, Basingstoke, Hampshire, UK), ii) modified M17 agar with 0.5% g/L glucose (M17 broth, Oxoid, Basingstoke, Hampshire, UK) and iii) solid sourdough bacterium medium (SDB) composed of 7.5 g/L maltose, 7.5 g/L glucose, 3.0 g/L yeast extract, 0.3 mL/L tween80, 6 g/L tryptone, 500 ml/L yeast water, 500 mL/L distilled water, agar 15 g/L at pH 5.6. LAB counts were determined after 48 h of incubation at 30 °C under anaerobiosis. The growth of

acetic acid bacteria (AAB) was evaluated on glucose-yeast extract-calcium carbonate (GYC) agar medium prepared as reported in Du Toit and Lambrechts (2002) with some modification: (50 g/L glucose, 10 g/L yeast extract, 5 g/L CaCO3, and 20 g/L agar) and incubation for 96 h at 30 °C. Media used for LAB and AAB plating were supplemented with 0.1 g/L of cycloheximide (Sigma-Aldrich) (Minervini et al., 2015). Enterococci were enumerated on Slanetz Bartley agar 37 °C for 48h (Oxoid, Basingstoke, Hampshire, UK). Staphylococci were counted on Baird Parker modified with egg yolk tellurite emulsion (50 g/L) (Oxoid, Basingstoke, Hampshire, UK), after 24h at 37 °C. Enterobacteriaceae were counted on Violet Red Bile Glucose (VRBG) agar medium (Oxoid, Basingstoke, Hampshire, UK), after 24h of incubation at 37 °C. The number of *Bacillus* was estimated after 24h of incubation at 37 °C by using LBG medium (10 g/L tryptone, 10 g/L yeast extract, 10 g/L NaCl, 1 g/L glucose, 15 g/L agar) at pH 7.0 (De Angelis et al., 2021). The number of yeasts and molds was estimated at 30 °C by using Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, Hampshire, UK), supplemented with chloramphenicol (0.1 g/L) (Sigma-Aldrich) for 48h. Plate counts were performed in duplicate.

2.4. Total microbial genomic DNA extraction

A total of 20 samples (liquid samples at D0 were excluded) were inspected by a metabarcoding analysis. Sample DNA was extracted from 5 g of doughs at three times: prior to fermentation (D0), after the first 24h of fermentation (D24), and after 10 (R10) days of sourdough propagation. Preliminary steps (Minervini et al., 2010) were followed prior to DNA extraction obtained by using the FastDNA Soil Kit (FastDNA Spin Kit for Soil, MP Biomedicals, Italy), according to the manufacturer's instructions.

Extracted DNA was eluted in 100 μl of purified water and stored at −20 °C. Quality and quantity of DNA extracts were estimated using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, US) and by 1% (w/v) agarose gel electrophoresis in TAE buffer.

2.5. Library preparation and sequencing

Total DNA extracted from dough samples was used as a template for 16S metagenetic analyses, based on the 350F/814R primer couple targeting the V1–V3 region of Firmicutes (Mühling et al., 2008). PCR were run at Genomix4Life S. r.l. (Baronissi, SA, Italy), using customized protocols. The same company provided for PCR product sequencing by using the Illumina 2 × 300bp paired end MiSeq platform.

16S sequencing-derived fastQ files were checked for quality using FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). In silico bioinformatics analyses, including denoising, taxa assignment and alpha and beta diversity, relied on the QIIME2 (Bolyen et al., 2019) microbiome platform (version 2020.8). More specifically, the QIIME plugin q2-deblur (https://github.com/qiime2/q2-deblur) was used for the 16S denoising step and Shannon entropy and Faith's PD were computed on the significance obtained using *ad hoc* available plugins. The SILVA 138 SSU database (https://www.arb-silva.de/documentation/release-138/) was used to infer the taxonomy starting from the ASV table. All the computed intermediate and final outputs not included, either as main documents or as Supplementary Material, are available upon request, and will be provided in the ".qzv" QIIME2 format.

2.6. Nucleotide sequence accession number

The sequences are available in the Sequence Read Archive of NCBI (Temporary Submission ID: SUB12614767).

2.7. Microbiota quantification by Real-Time PCR assays

Quantification of total bacteria and specific species was performed through qPCR on D0, D24 and R10 collected samples. Supplementary Table S1 shows the set of primers targeting the 16S rRNA gene and references used for the quantification of total bacteria and LAB involved in sourdough fermentation (Granger et al., 2008; Jomehzadeh et al., 2020; Kim et al., 2020; Pontonio et al., 2017; Sedgley et al., 2005; Stevenson et al., 2006). Primers used in this study were synthetized by Life Technologies Italia (Monza, Italy) under our specific request based on literature evidence. Real-time PCR were carried out using a range of microbial species to verify the specificity of each primer set. qPCR was performed on a 7300 Real-Time PCR System (Applied Biosystem, Forter City, CA USA). The reaction mixture (25 μL) contained 12.5 μl of SsoAdvanced™ Universal SYBR® Green Supermix, (BioRad), 0.1 μL of 0.2 μM of PCR primers, 11.4 μL of DNase and RNase-free water, and 1 μL of DNA sample (40 ng) (Table S1). The assays were carried out in triplicate. The amplification program consisted of 1 cycle of 95 °C for 1 s, 1 cycle of 95 °C for 2 s, followed by 40 cycles of 95 °C for 5 s, appropriate annealing temperature (Table S1) for 30 s and 72 °C for 35 s. After each amplification last cycle, a melting curve analysis, with a temperature ranging from 60 °C to 95 °C, was performed to ensure the specificity of the amplification products. The cycle threshold (C_T) was determined automatically by the instrument. qPCR amplification products were further loaded on agarose 2% gels to check the correspondence to amplicon size and further sequenced by Macrogene Europe (Milan, Italy).

The coefficients of efficiency (E) were calculated using formula $E = (10^{-1/slope}) - 1$ (Higuchi et al., 1993). For the absolute quantification, standard curves were constructed using genomic DNA of ten different LAB strains (*Fructilactobacillus sanfranciscensis; Levilactobacillus brevis; Latilactobacillus curvatus; Lactiplantibacillus pentosus; Lactobacillus acidophilus; Lactiplantibacillus plantarum; Limosilactobacillus fermentum; Pediococcus pentosaceus; Enterococcus mundtii; Enterococcus faecalis*) belonging to the culture collection maintained at DISSPA, Department of Soil, Plant and Food Sciences (University of Bari Aldo Moro, Bari, Italy) or purchased from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). 16S rDNA copy number was calculated as [CN = (DNA (ng) x 6.022×10^{23})/(length (bp) x 1 x 10^9 x 660)]. All strains were cultured in MRS medium, at 37 °C. Genomic DNA was extracted using Bacterial Genomic DNA Isolation Kit (Norgen, Thorold, ON, Canada), according to the manufacturer's instructions. Each DNA sample from reference strains was serially 10-fold diluted and the concentration was confirmed by NanoDrop spectrometer assay. Diluted DNA was used in qPCR reaction as above. For each experiment, a negative control (NTC, no template control) was used. All the assayed negative controls showed the absence of amplification. The standard curves were constructed from 10-fold serially diluted reference strains DNA at known copy number, covering a dilution range of 6 orders (10^3–10^9), the resulting C_T were plotted against the logarithm of the 16S copy number. The copy number of 16S gene of each species was calculated in dough and sourdough samples by comparing the C_T value with its mate in the standard curve. The amplification efficiencies of all qPCR assays ranged from 91% to 98%.

2.8. Community level physiological profiling

Carbon source utilization patterns of microbial communities in the 24 samples (Table 1) were assessed by using BIOLOG 96-well Eco-plates (Biolog, Inc., Hayward, CA, USA) according to (Cavallo et al., 2017). Microplates contained 31 different carbon sources (carbohydrates, carboxylic acids, polymers, amino acids, amines and miscellaneous substrates), in triplicate. Ten grams of each sample were homogenized in 90 ml of sterile NaCl solution (0.9% w/v) in a stomacher bag (Bag Mixer, Interscience International, Roubaix, France) for 3 min. Subsequently, the homogenate sample was centrifuged at 10.000 rpm for 15min at 4 °C and the relative pellet was resuspended (1:10) in Tris-HCl 50 mM at pH 7.5. Two other steps of centrifuge and resuspension pellet in NaCl

solution (0.9% w/v) were performed to remove interfering residuals. The final suspension was inoculated on Biolog ECO-plates, which was incubated at 30 °C in the dark, and plates were analysed at 590 nm for three times (each 24 h) with a microplate reader (BIOLOG Microstation).

2.9. Water-soluble and methanol-soluble extract preparation

Water-soluble extracts (WSE) from D0, D24, and R10 samples were prepared according to the method originally described by Osborne (1907, pp. 1–132) 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 biochemical analyses. Methanol-soluble extracts preparation started from 5 g of each sample that were mixed with 20 mL of 80% methanol to get methanolic extracts (MEs). The mixture was purged with nitrogen stream for 30 min, under stirring condition, and centrifuged at 4600×*g* for 20 min. The supernatants, corresponding to MEs, were transferred into test tubes, purged with nitrogen stream and stored at ca. 4 °C before analysis.

2.10. Biochemical characterization

The pH value of doughs and sourdoughs (before and after fermentation) was determined by a pH meter (Model 507, Crison, Milan, Italy) with a food penetration probe at the beginning and end of fermentation. ΔpH was calculated as the difference between pH values at the beginning and at the end of fermentation (Lattanzi et al., 2013). Total titratable acidity (TTA) was determined at 0 h and after fermentation by homogenization of 10 g of dough with 90 ml of distilled water for 3 min in a stomacher bag (Bag Mixer, Interscience International, Roubaix, France), titrated with 0.1M NaoH and expressed as the amount (ml) of 0.1 M NaOH needed to titrate the solution to pH 8.5.

The content of lactic acid and acetic acid was determined with commercial kits, K-DLATE and K-ACET (Megazyme, Wicklow, Ireland) kits. The quotient of fermentation (QF) was determined as the molar ratio between lactic and acetic acids. The peptide concentration in WSE was determined by the *o*-phthaldialdehyde (OPA) method (Church et al., 1983; Perri, Coda, et al., 2021). A standard curve prepared using tryptone (0.25–1.5 mg ml−1) was used as the reference. The total amino acids were quantified with the ninhydrin test (Friedman, 2004).

2.11. Nutritional characterization

Total phenols were determined on the methanolic extracts (MEs) of doughs. The concentration was determined as described by Slinkard and Singleton (1977) and expressed as gallic acid equivalent. MEs were used to determine the antioxidant properties. The free radical scavenging capacity of MEs was determined using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) evaluating the ability of sourdoughs to act as a scavenger against the stable chromogenic radical DPPH following the decrease in absorbance at 517 nm as reported by Yu et al. (2002). The DPPH solution was prepared at 0.08 mM concentration in absolute ethanol. The absorbance value was compared with 75 ppm butylated hydroxytoluene (BHT) as the antioxidant reference.

For the determination of the content of phytic acid and total phosphorus, the Phytic Acid (Phytate)/Total Phosphorus kit (K-PHYT 05/19; Megazyme, Wicklow, Ireland) was used, following the manufacturer's instructions.

Raffinose concentration was determined using the Raffinose/D-Galactose kit (K-RAFGA 06/20; Megazyme, Wicklow, Ireland), according to the manufacturer's instructions.

2.12. Statistical analysis

When appropriate, based on different metadata sample stratifications, a two group Welch corrected test (Bejamini-Hochberg) was used to perform group pair comparisons and retrieve statistically significant corrected variables. Results were graphically rendered by means of proportion graphs. Specifically, based on the 95% of the distribution confidence interval, we plotted the difference in mean proportions (%) of the two groups.

All performed analyses were corrected for multiple tests by applying the Benjamini-Hochberg procedure. The mean proportion graphs included error bar plots have been obtained by using the STAMP software (Parks et al., 2014). The alpha diversity was computed based on the Faith's PD metric and plotted as interquartile boxplots. Looking for statistically significant changes in alpha and beta diversity estimates starting from Bray-Curtis, Jaccard and Unweighted UniFrac computed distance matrices, the QIIME II Emperor nested plugin was used to plot the PCoA results. The PERMANOVA test was also computed in QIIME2 environment to obtain intergroup beta diversity significance.

3. Results

We here investigated biochemical and microbiota taxa profiles in four different flour matrices, inclusive of sprouted and non-sprouted samples, that were spontaneously propagated until the 10th backslopping (every 8 h) in both solid and liquid conditions. The matrices precisely accounted for raw wheat, non-sprouted whole wheat, sprouted whole wheat and a mix made of sprouted whole wheat and sprouted whole lentils.

Different grouping stratification were tested based on the above-mentioned features and replicates were also inspected based on time of refreshment at the starting point (D0), after the first day of backslopping at 30 °C (D24) and after the 10th day of refreshment at 30 °C (R10). Table 1 reports the considered matrices together with the different tested group stratifications.

3.1. Biochemical and nutritional features compared at different refreshment timepoints

Biochemical (pH, total titratable acidity, lactic acid, acetic acid, fermentation quotient, total free amino acids, peptides) and nutritional characterization (total phenolic concentration, antioxidant activity and antinutritional factors (phytic acid and raffinose)) of dough and sourdoughs at D0, D24 and R10, were carried out and the relative average values (plus/minus standard deviation) have been summarized in the Supplementary Table S2.

When biochemical features were compared by inspecting the refreshment time points (two group corrected Welch test) no statistically significant value emerged.

3.2. Cell density at different refreshment timepoints

By inspecting the media count values at different timepoint refreshment, regardless of neither the matrix belonging nor the sprouting condition, LAB followed a statistically significant increase from D0 to both D24 and R10 refreshments (Fig. 1). On the contrary, the total cell density of presumptive Enterobacteriaceae were absent at R10.

Presumptive yeasts significantly increased at R10 when compared with D24 (Fig. 1C).

3.3. Firm and liquid conditions

The same variables whose measurements have been reported in Supplementary Table S2 were statistically compared in terms of firm and liquid group belonging. The Welch test paired group comparative results (DY_160 vs. DY_280) have been shown in Table 2 and are refereed only to statistically significant hits. More precisely, the presented table lists the mean relative frequency values plus/minus standard deviation.

Before the fermentation process started (D0), the pH resulted to be significantly increased in the liquid samples ($q < 0.05$). Oppositely, at

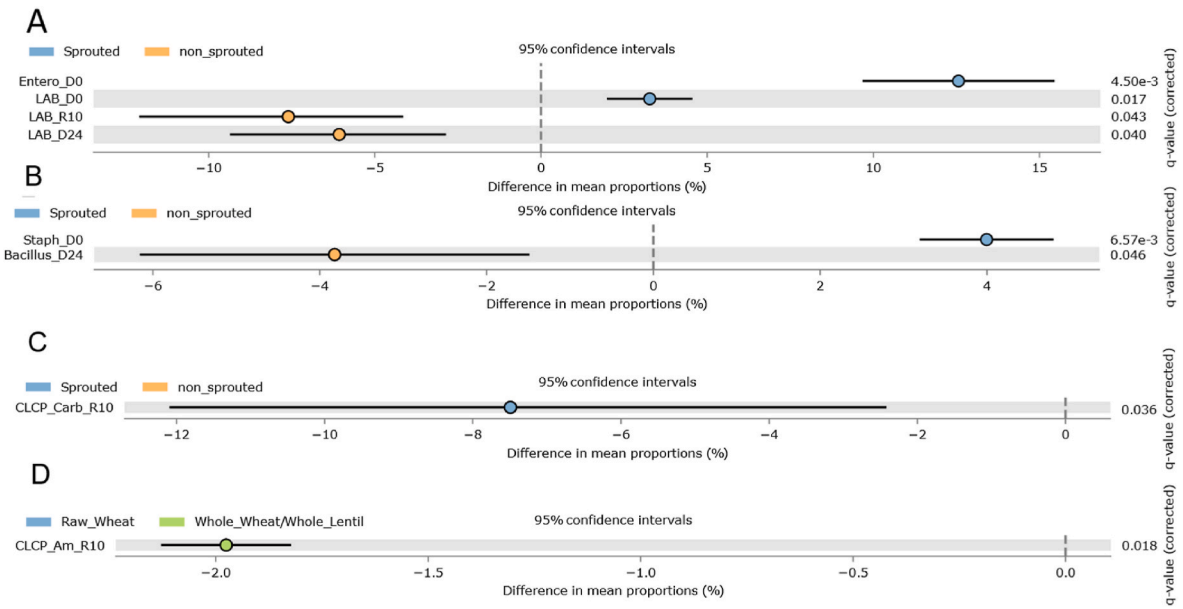

Fig. 1. Two group Welch corrected test of qPCR taxa based on time stratification. Difference in mean proportions from a two group Welch corrected comparison (BH) was computed based on qPCR cell densities (log CFU g^{-1}) using time as stratification variable (D0, D24 and R10). Only statistically significant results were reported in terms of mean proportions. Figure panels are relative to D0 versus D24 (panel A), D0 versus R10 (panel B), and D24 versus R10 (panel C). For each statistically significant taxa the relative table reports the percentage of the mean relative frequency in both the compared groups, the standard deviation, the p-value, the correction for multiple test (p-value corrected), the difference between means, and the lower and the upper confidence interval (CI) relative to the 95% of the total distribution.

Table 2
Two group corrected Welch's test run on physiochemical and nutritional variables in firm and liquid samples. The comparison of the two dough yield groups resulted in statistically significant variables including pH, total titratable acidity (TTA), phytic acid (PA), and raffinose content (RAF) measured at D0 (prior to the fermentation) and after the first 24h of fermentation (D24) before the beginning of daily propagations. Values were expressed mean proportions±their relative standard deviations. Only statistically significant variables (q-values) were reported.

	DY_160	DY_280	q-values
pH_D0	38.76 ± 0.71	42.39 ± 0.86	0.0045
TTA_D0	18.73 ± 1.51	11.64 ± 2.83	0.0432
PA_D0	49.81 ± 0.51	52.12 ± 1.25	0.0418
PA_D24	46.22 ± 1.42	43.41 ± 1.08	0.0369
RAF_D0	49.50 ± 0.67	52.07 ± 0.63	0.0087
RAF_D24	46.08 ± 1.21	43.60 ± 0.81	0.0456

the same time point, the total titratable acidity (TTA) value, resulted to be increased in the firm samples. Statistically significant higher amounts of raffinose (RAF) and phytic acid (PA) content were found in liquid samples (DY280) at D0. On the contrary at D24 an increase of both RAF and PA was found in firm samples (DY160).

3.4. Biochemical values impacted matrix and sprouting group stratification

To better detailing the impact of the different grain composition and the sprouting process, we inspected the gather physicochemical value matrix by stratifying samples according both to sprouting and matrix group stratifications. Statistically significant results are reported in Table 3.

pH values at R10 appear significantly higher in sprouted samples than non-sprouted ones (Table 3).

RAF at the 10th refreshment increased in non-sprouted samples.

When the matrix stratification was used in a two-group Welch corrected test the fermentation quotient (FQ) was found to be significantly

Table 3
Statistically significant physicochemical variables resulted from two group Welch comparison. Corrected Welch test statistically significant variables between sprouted/non sprouted and matrix type (indicated as matrix 1 and matrix 2) included pH, fermentation quotient (FQ), lactic acid (LA), and raffinose content (RAF). Values were expressed as mean proportion from Welch's corrected test±their relative standard deviations. p-values were corrected by multiple tests using Benjamini-Hockberg procedure.

	Group 1	Group 2	
Physicochemical parameter	Sprouted	Non-sprouted	q-values
pH_R10	28.59 ± 0.28	26.17 ± 1.14	0.0313
RAF_R10	4.00 ± 0.30	4.74 ± 0.20	0.0171
	RW	**SWSL**	
FQ_R10	43.77 ± 2.55	21.55 ± 1.28	0.0357
	RW	**SW**	
FQ_R10	43.77 ± 2.55	26.49 ± 6.47	0.0043
	SW	**SWSL**	
LA_D0	16.83 ± 1.73	12.98 ± 0.11	0.0027

increased at R10 in raw wheat (RW) samples compared both to the mix (SWSL, q = 0.03) and the sprouted wheat (SW, q = 0.004).

Moreover, lactic acid (LA) amount was found to be significantly higher in SW than SWSL at D0.

The sprouting process impacted taxa composition and metabolism as supported by data shown in Fig. 2. In details, the two group statistical comparison results are relative to the difference in qPCR taxa abundance (Fig. 2 panel A–B), and in the community level physiological profiling (CLPP), relative to the bacterial usage of amino acids, carbohydrates, carboxylic acids, amines and polymers (Fig. 2 panel C–D).

In detail, culture dependent presumptive lactic acid bacteria (LAB) cell densities increased after 24h of fermentation and increased more at the end of the 10th refreshment in the non-sprouted matrices (Fig. 2A). In fact, percentage values relative to the difference in mean proportion (the difference between sprouted and non-sprouted values) also evidenced how presumptive Staphylococcus density significantly increased in sprouted samples at D0, whereas Bacillus were higher in non-sprouted

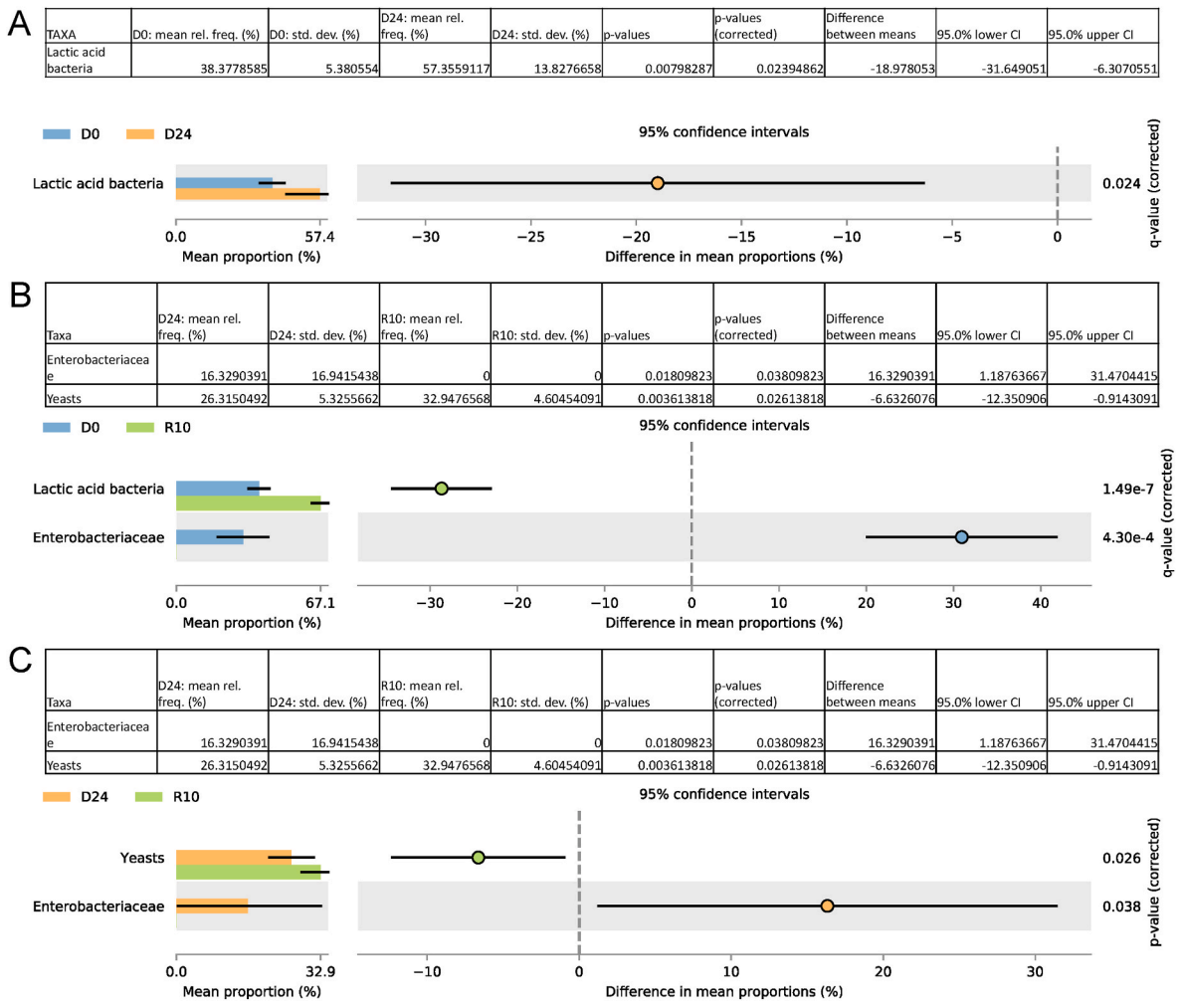

Fig. 2. Difference in mean proportion from a two group Welch corrected comparison (BH) between sprouted and non-sprouted samples. Statistically significant two group Welch test (non-sprouted versus sprouted) in culture-dependent microbiological taxa at D0, D24 and R10 (Panel A–B); carbohydrate metabolism from the analysed community level physiological profile (CLPP) (Panel C). Panel D reports the only statistically significant amino acids CLPP (Am) emerging from non-sprouted raw-wheat (RW) and sprouted wheat/lentil (SWSL) comparison, at R10. After testing all the possible stratifications, i.e. dough yield, sprouting process, and matrix belonging, only statistically significant results were reported as the difference in percentage of mean proportions.

samples at D24 (Fig. 2B).

When CLPP profiles were checked, the sprouted matrices revealed an increase in the metabolisms related to carbohydrates at the 10th refreshment timepoint (CLPP_Carb_R10 in Fig. 2C). Furthermore, when compared with RW, the SWSL matrix showed a higher amino acid metabolism profile (Fig. 2D).

3.5. Metataxonomic profile of raw wheat and sourdough

To obtain the metataxonomic profiles of raw wheat, whole wheat and mixed samples, the total DNA extracted from doughs at D0, after the first 24 h of fermentation (D24), and after 10 days of propagation (R10) under firm (F) and liquid (L) conditions was sequenced by meaning of high throughput approach. The V1–V3 hypervariable target region of the 16S rRNA gene was sequenced on the MiSeq platform, using a 2 × 250 bp paired-end (PE) sequencing strategy. Raw sequencing data are publicly available as bioproject within the SRA repository under the following Temporary Submission ID: SUB12614767. Retained PE reads, as ASVs (Amplicon Sequence Variants), following the denoising procedure have been reported in Supplementary Material.

The Faith's PD alpha diversity index reported in the Supplementary Fig. S2 as boxplot distribution was not meaningful of a significant difference of taxa distribution between firm (DY160) and liquid (DY280) samples (data not shown). Instead, when the flour matrices were used to stratify samples both the multiple corrected Kruskal-Wallis test (based on Faith's PD) and the PERMANOVA analysis (based on Bray-Curtis distance matrix), revealed statistically significant differences of alpha and beta diversity estimated between these groups. More precisely, the Kruskal-Wallis highlighted significant difference between RW and both the SW and SWSL matrices (Supplementary Table S3).

Except for NSW/SW comparison all the other tested two-group statistical results analysed by using the PERMANOVA test indicated a statistically significant change in alpha diversity values among matrix groups (Supplementary Table S4). Microbial diversity was also evaluated using the unweighted UniFrac metric and rendered as a principal coordinate analysis graph (Supplementary Fig. S3) where, although the tridimensional plot describes approximately the 70% of the total distribution, no cluster separation emerged based on dough yield group belonging (Fig. S3A) or propagation time (data not shown).

On the contrary, the same analysis slightly differentiated samples based on matrix belonging (Fig. S3B).

Taxa abundance profiles were used to compute statistically significant differences between groups and few significant evidence ($q < 0.05$) emerged at the genus level.

Briefly, as highlighted by the extended error bar plots, the sprouting process drives a significant presence of the genus *Pediococcus* that

significantly increases in sprouted samples (Fig. 3A). This was also confirmed when the SWSL was compared both against the NSW and RW group (Fig. 3 B, 3C).

The sprouting and the fermentation ongoing process, measured at the 10th day of refreshment, led to an increase in *Weissella* and *Empedobacter* genera as emerged when non-sprouted wheat versus mix, and the non-sprouted wheat D24 versus R10 were compared, respectively (Fig. 3B and D).

3.6. Real time PCR absolute quantification

To better inspect the taxa profile of actively transcribing bacteria at the species level and obtain an absolute quantification of the tested alive species, we used a qPCR culture-independent approach. Totally, ten species of bacteria selected from literature, according to culturomics and metataxonomic genus experiments, were analysed. Calibration curves were used to assess the absolute abundances of these species in dough before the first fermentation (D0), and in sourdough after 24h and 10 days of propagation. By interpolating the threshold cycle (CT) on the standard curves, the bacterial quantities were determined and expressed as Log (Copy Number) value (Supplementary Table S5). As show in Supplementary Fig. S4, when raw wheat was compared with both sprouted wheat (SW) and sprouted wheat/sprouted lentils (SWSL), *Enterococcus faecalis* and *Pediococcus pentosaceus* species increase in sprouted samples. Among other significant log copy number changes *Enterococcus mundtii* was significantly higher in raw wheat compared both to non-sprouted wheat and SWSL mix. On the other hand, sprouted wheat has a higher amount of this taxa when compared with NSW.

4. Discussion

Previously published studies reported the use of germinated flours in bread (Al-Ansi et al., 2022; Atudorei et al., 2022; Guardado-Félix et al., 2020) and sourdough bread formulations (Montemurro et al., 2019; Perri et al., 2020). The usage of sprouted grains made from cereals and legumes as an alternative ingredient useful in obtaining health-promoting foods is worldwide increasing. Sprouted grain nutritional profiles proved to contain micro- and macro-nutrients together with bioactive compounds that actively support the spread of extra nutrient enriched foods (Benincasa et al., 2019). To date, the impact of different sprouted grain flours on the type I sourdough microbiological and nutritional properties have been not evaluated yet, even on a dough yield basis. As previously reported, type I sourdough were studied as closely linked to leavened baked good production (Arora et al., 2021; Di Cagno et al., 2014; Ercolini, 2013; Minervini et al., 2012; Rizzello et al., 2014). We here inspected the ecological dynamics of four traditional type I sourdoughs, propagated under firm (DY160) and liquid (DY280) conditions by using i) a refined wheat, ii) a whole non-sprouted wheat, iii) a whole sprouted wheat, and iv) a blend of sprouted wheat and sprouted lentil.

Our approach allowed for the evaluation of the commensal microbial community, using culturomics, community catabolic profile, qPCR assay and 16S rRNA gene metataxonomic analysis. Also, we investigated the physicochemical and nutritional properties of dough and type-I sourdough in terms of pH, TTA, lactic acid, acetic acid, fermentation quotient, total free amino acids, peptides content, total phenol content, antioxidant activity, raffinose content, phytic acid, at different collected time.

Among physicochemical parameters, pH and TTA values resulted to be sensible to dough yield condition and in turn lead to a change in the microbial diversity (De Vuyst et al., 2014; Minervini et al., 2014). In line with this, before the fermentation started, we observed higher pH and lower TTA values in liquid samples when compared with firm ones.

Moreover, despite not supported by statistical significance, we

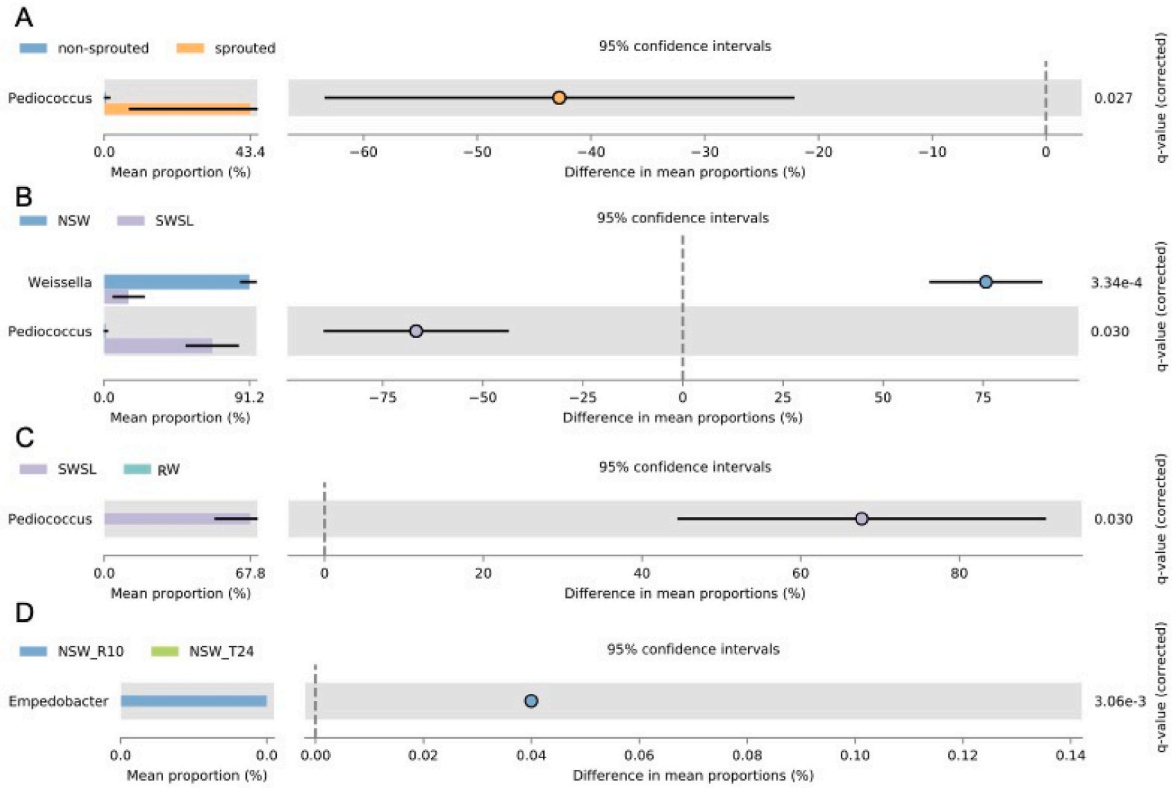

Fig. 3. Two group Welch comparison on microbiota profile. Statistically significant 16S taxa have been shown based on all possible grouping stratifications (sprouting, matrix type and time) as difference in mean proportions between a) non-sprouted and sprouted, b) non sprouted wheat (NSW) and sprouted wheat/lentil mix (SWSL), c) SWSL and raw wheat (RW), and d) NSW at D24 and at the last refreshment (R10).

noticed a decreasing pH trend for liquid sourdoughs till the end of the fermentation process with pH values lower than 4.5. This trend is in line with previous evidence (Di Cagno et al., 2014), stating how liquid sourdoughs obtained only from raw wheat showed lower pH values at the end of the fermentation.

A sprouting-dependent increase in the total phenolic content (TPC) drives towards an increase in antioxidant capacity in doughs samples (D0). This increase is most probably linked to most bio-accessible-free form of polyphenols and extractable bioactive components present in wheat seeds (Alvarez-Jubete et al., 2010), which does not include the refined raw wheat RW. At the same, polyphenols highest values were detected in firm sourdoughs; this agrees with the observed lower pH and higher TTA values, indicating higher concentrations of acidic compounds in firm than in liquid samples. This may be responsible for higher observed antioxidant activities. Albeit not statistically significant, a close linear correlation was present between sprouting, TPC and antioxidant activity at the end of propagation. The comparable LAB dominance at the end of fermentation, lead to a specific capacity based on esterase activity and the property to hydrolyze complex phenolic compounds and their glycosylated forms into their corresponding phenolic acids. This could break down the differences detected in firm and liquid samples (Nionelli et al., 2014).

By inspecting sprouted group stratification, sprouted samples are marked by significantly higher pH values at the 10th refreshment. This evidence could be explained by the lower presence of LAB detected by culturomics analyses in sprouted samples probably due to the presence of wheat (Perri et al., 2020).

Moreover, the higher detected pH value could be explained based on the comparison of stratified samples based on the group matrix belonging. Specifically, when SW and SWSL were both compared against RW, a lower amount of organic acids (acetic and lactic) marked the sprouted samples, as also supported by the lower fermentation quotient measured at the end of the fermentations.

As a consequence of minimal endogenous phytase activity in non-sprouted cereal and legume grains, these samples were enriched in phytic acid (Gupta et al., 2015). However, we did not found a statistically significant decrease trend in phytic acid in our sprouted samples at the end of fermentation.

As evidenced by literature, the combination of sprouting process and sourdough technology could be an excellent strategy to improve nutritional and functional features of sourdough directly impacting human health (Diowksz et al., 2014; Galli, Venturi, Pini, Guerrini, & Granchi, 2019). In this study, the lower raffinose amount in sprouted samples, a fermentable sugar included in the class of fermentable oligosaccharides, disaccharides, monosaccharides and polyol (FODMAP), would prevent gastrointestinal symptoms (Curiel et al., 2015; Gangola et al., 2014). At the same, phytic acid that chelates divalent minerals and reduces their bioavailability, was detected at lower concentrations in sprouted and mature sourdough (Curiel et al., 2015; Yildirim & Arici, 2019).

Although we found only a lowering trend describing the phytic acid decrease at end of fermentation, our data revealed a statistically significant lower value of raffinose in sprouted than non-sprouted samples.

It is known that different strains of lactic acid bacteria (LAB) belonging to *Lactobacillus* genus, can produce in sourdough the *α-Galactosidase* enzyme useful to reduce the raffinose family oligosaccharides (RFO's) content in different products (Yoon & Hwang, 2008). qPCR results detected several species of lactobacilli whose presence could justify raffinose level reduction in mature sourdoughs (R10).

The cell density of the main microbial groups was affected by matrix, time of collection and condition of propagation. In line with previously published papers (Perri et al., 2020; Y. Yang et al., 2013) our data evidently supported by statistics, showed how LAB cell densities decreased at D24 and R10 in doughs made by flours obtained from sprouted grains.

The yeast initial cell density increases during propagation till the 10th daily refreshment. Sprouted sourdoughs harboured a higher number of yeasts. A similar trend was previously observed in spontaneous fermentation of sprouted lentil flour alone or in mix with cereal flours (Perri et al., 2021a; Perri et al., 2021b). According to Ercolini and co-workers (Ercolini, 2013), Enterobacteriaceae were almost completely inhibited in all sourdoughs at the end of fermentation.

Microbial community structure, the influence of the ingredients, process parameters, and the house microbiota in sourdough ecosystems have been studied intensively in the last few decades, also using high-throughput sequencing technology which, inspected the relative abundance until the taxon level, giving the opportunity to obtain an overview on the community composition (Comasio et al., 2020; De Filippis et al., 2018; De Vuyst et al., 2014; Minervini et al., 2014; Van Kerrebroeck et al., 2017). As evidenced by alpha and beta diversity estimates, we noticed how statistics proved a different distribution of taxa based on different metadata. Precisely, intergroup beta diversity described a better clustering of samples when the matrix stratification was used instead of that based on doug yield.

A higher relative abundance of *Pediococcus* genus was found in the SWSL blend when metataxomic data were inspected. *Pediococcus* presence was evaluated as an additional measure for the improvement of product safety by Rossi and Lathrop (ROSSI & LATHROP, 2019) that tested a blend of bacteria composed of two *Pediococcus* species on alfalfa sprouted seeds and proved to reduce the growth of pathogens including Listeria monocytogenes and Salmonella.

At the species level NSW was found to be significantly enriched in *Empedobacter* a species potentially useful to enhance the wheat tolerance to drought stress (Hasanuzzaman et al., 2018).

A significantly increased relative abundance of Weissella in NSW was also found in line with (Hu & Gänzle, 2018). Although some literature evidence, flagged Weissella as an opportunistic pathogen a highlighted the impossibility to classify some taxa as "generally regarded as safe" (GRAS) (Abriouel et al., 2015), this genus includes at the species level some other taxa that are capable to produce exopolysaccharides (EPS) (Kavitake et al., 2020). EPS are widely used in food industries as thickeners, stabilizers, emulsifier, and texture improvers. Moreover, EPS can improve human health due to its antioxidant, antitumor, antiulcer, immunomodulating or cholesterol-lowering activities (Adebayo-Tayo et al., 2018).

Significant abundance of *Enterococcus faecalis* emerged from the comparison of both SW and SWSL against RW. This results on sourdough is in line with our previous evidence on SW germinated flour Perri et al. (2020). *Enterococcus faecalis* exerts an important role in the production of γ-aminobutiric acid (GABA) (Khanlari et al., 2021), an aspect that deserves further investigations (de Almeida et al., 2018).

Moreover, although marked by low copy number values in qPCR, *Enterococcus mundtii* abundance significantly differed in RW when compared with NSW and SWSL, and in SW when compared with NSW samples. Feng and colleagues argued about a possible increase of *Enterococcus mundtii* as a consequence of the sprouting condition in alfalfa seeds (Feng et al., 2009). A concomitant decrease in phytic acid and a parallel increase of gliadin degradation has been associated to its presence (Sakandar et al., 2018). Lastly, the increased amino acidic and carbohydrate CLPP profiles that marked sprouted samples, indicated their higher enzyme activity and microorganism metabolism as reported by Diowksz and colleagues (Diowksz et al., 2014; Grassi et al., 2018). Thus, sprouted and fermented foods have shown hypoglycaemic effects on humans and animals, most probably due to reduced concentrations of soluble carbohydrates, increased dietary fibre levels and resistant starch (Ikram et al., 2021; Lopes et al., 2019).

The here reported results highlighted how the propagation under firm and liquid condition was influenced by sprouting process as revealed by the physicochemical and taxonomic profiles of samples.

5. Conclusions and future perspectives

The synergistic cooperation of bacterial and yeast taxa in sprouted

grains needs to be tested by using a higher number of samples which, in fact, is a limitation of this study. To better understand the impact of sourdough variable microbiological and physicochemical composition, a comparative assessment of bakery products at the end of the step-by-step company procedures are required. Further, experimental evaluations based on metatranscriptomics analyses are required to test the effective consortium metabolic pathways in terms of transcribed functions.

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Data statement

The obtained 16sRNA fastQ sequences are available within the NCBI Bioproject database (Temporary ID: SUB12614767). All the produced data have been included in the paper body or in the related supplementary material.

CRediT authorship contribution statement

Maria Calasso: Conceptualization, Project administration, Methodology, Writing – review & editing. **Arianna Ressa:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Francesco Maria Calabrese:** Methodology, Data curation, Supervision, Writing – original draft, Writing – review & editing. **Fabio Minervini:** Methodology, Investigation, Formal analysis. **Maria De Angelis:** Conceptualization, Resources, Funding acquisition, Supervision.

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.

Data availability

All data have been shared

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115285.

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