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Sourdough fermentation for the valorization of sorghum flour: Microbiota characterization and metabolome profiling

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ABSTRACT

Due to a large adaptability to different cultivation conditions and limited input compared to other cereals, sorghum is considered an emerging crop. Its antioxidant properties, high fiber content and low glycemic index also make it a valuable addition to a healthy diet, nevertheless, the presence of antinutritional factors and the lack of gluten, hamper its use as food ingredient. This study investigated the impact of sourdough fermentation on sorghum nutritional quality. Lactic acid bacteria dominating sorghum flour and sourdough were identified by culture-dependent analysis revealing *Lactiplantibacillus plantarum* as the dominant species found in the mature sourdough, whereas *Weissella cibaria* and *Weissella paramesenteroides* were the species isolated the most after the first refreshment. Among yeasts, *Saccharomyces cerevisiae* was the most prevalent. Lactic acid bacteria protechnological and functional performances as starter were evaluated in sorghum type-II sourdoughs through an integrated characterization combining chromatographic and NMR spectroscopic techniques. The metabolic profile of the strains mainly grouped together *W. cibaria* strains and *W. paramesenteroides* AI7 which distinguished for the intense proteolysis but also for the presence of compounds particularly interesting from a physiological perspective (allantoin, glutathione, γ-aminobutyric acid and 2-hydroxy-3-methylbutyric acid), whose concentration increased during fermentation in a species or strain specific matter.

1. Introduction

The intensification of agriculture practices relying on external inputs (fertilizers and pesticides), cropland expansion, and the cultivation of only a few selected cereal species or varieties, particularly emphasized in the last few decades, had dire outcomes on the environment. This approach progressively led to the abandonment of minor and local crops, with severe consequences on genetic biodiversity preservation and local economies. However, within the current climate change crisis, these underutilized cereals are under large reconsideration due to their agronomic and nutritional value. These cereals still represent the main staple food in some regions, and include einkorn, emmer, millet, oats, rye, spelt, sorghum, teff, triticale, and tritordeum (Gazza and Nocente, 2023).

Among them, sorghum (*Sorghum bicolor* L.), often used as feed, is becoming increasingly important for its positive agronomical impact. Sorghum can adapt to various conditions and is particularly resistant to limited input compared to other cereal crops (Hossain et al., 2022). Due to its ability to withstand high salinity and low water supply it is well suited to marginal areas where both conditions are more prevalent. Moreover, its antioxidant properties, high fiber content and low glycemic index make it a valuable addition to a healthy diet (Hossain et al.,

Abbreviations: DY, dough yield; FAA, free amino acids; fS, fermented sorghum; fS_Lp_AI4, sorghum fermented with *L. plantarum* AI4; fS_Lp_T0I5, sorghum fermented with *L. plantarum* T0I5; fS_Lp_T10I, sorghum fermented with *L. plantarum* T10I1; fS_Lp_T10I3, sorghum fermented with *L. plantarum* T10I3; fS_Lp_T10I5, sorghum fermented with *L. plantarum* T10I5; fS_Wc_T1I4, sorghum fermented with *W. cibaria* T1I4; fS_Wc_T1I8, sorghum fermented with *W. cibaria* T1I8; fS_Wp_AI7, sorghum fermented with *W. paramesenteroides* AI7; LAB, lactic acid bacteria; mMRS, modified De Man Rogosa Sharp; rS, raw sorghum; SDA, sabouraud dextrose agar; TTA, total titratable acidity.

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2022). Nevertheless, few aspects hamper its use as food ingredient. Being a gluten-free cereal, sorghum application in gluten-containing foods can be deleterious for their structure. Moreover, its antinutritional factors (e.g., tannins, phytate, enzyme inhibitor), responsible for the limited protein and minerals bioavailability, hinder its use as food ingredient (Rashwan et al., 2021). Compared to other cereals, sorghum has a higher content of polyphenols, with phenolic acids, flavonoids, condensed tannins, and deoxyanthocyanidins being the predominant compounds (Khoddami et al., 2023). Polyphenols content is affected by genetic and environmental factors, such as plant color, thickness of the pericarp, and growth conditions. For instance, grains color can vary, among several cultivars, from white to red, with red-colored grains having higher total polyphenol content. Tannins, also causing the bitter and astringent taste, are scarcely present in white sorghum cultivars, that are the most suitable for food use (Khoddami et al., 2023). Sourdough fermentation was overall proven to be effective in improving the nutritional, sensory, functional, and technological properties of cereals, including ancient and minor cereals (Pontonio et al., 2023). For instance, it was demonstrated that fermentation of sorghum with a combination of lactic acid bacteria strains can contribute to polyphenols metabolisms, through glucosidase, phenolic acid reductase, and phenolic acid decarboxylase activities thus influencing sorghum nutritional value (Svensson et al., 2010). Partial or complete elimination of tannins, phytic acid, protease inhibitors, and α-galactosides was reported for many of these cereals as consequence of lactic acid bacteria fermentation (Pontonio et al., 2023), yet, to fully exploit the potential of such matrices, when setting up a fermentation process the starter selection is essential to guarantee its success.

In this framework, the effects of fermentation on sorghum can be of pivotal importance to fully exploiting its potential as ingredient, thus contributing to the sustainability of the entire food system. Therefore, the aim of this study was to investigate the impact of lactic acid fermentation on sorghum. A traditional sourdough was obtained by spontaneous fermentation followed by a backslopping procedure and characterized. Lactic acid bacteria (LAB) and yeasts dominating sorghum flour and sourdough were identified by culture-dependent analysis, and then their pro-technological and functional performances as starter evaluated in sorghum type-II sourdoughs. An integrated characterization including a metabolomic approach combining chromatographic and NMR spectroscopic techniques was applied.

2. Materials and methods

2.1. Raw materials

Organic white sorghum (*Sorghum bicolor* L.) flour (EcorNaturaSì SpA, Treviso, Italia) having the following proximal composition: carbohydrates, 66 g/100 g; fiber, 7.5 g/100 g; protein, 9.3 g/100 g; fat, 3.2 g/100 g; ashes, 2.1 g/100 g was used in this study.

A type 0 wheat flour (Coop, Casalecchio di Reno, Italia) having the following proximal composition: moisture, 14.5 %; proteins, 11.75 % on dry matter (d.m.); carbohydrates, 83.4 % on d.m.; dietary fibers 2.9 % on d.m.; fat, 1.2 % on d.m.; ash, 0.7 % on d.m. was used to assess yeasts leavening potential.

2.2. Sourdough preparation and propagation

A sorghum sourdough was prepared and propagated through a protocol, traditionally used for wheat sourdough, without the use of starter cultures or baker's yeast (De Vuyst et al., 2023). Flour was mixed with tap water at a final dough yield (DY) [dough weight × 100/flour weight] of 160, corresponding to a flour:water ratio of 62.5:37.5. The first fermentation was carried out at 30 °C for 24 h. Afterward, daily backsloppings were performed for 10 days, mixing 25 % of the previously fermented dough with flour and water (final dough yield of 160), and incubating at 30 °C for 8 h. For the analyses, aliquots of sourdoughs were also taken at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 e 10 days of propagation (T0, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10). Sourdough backsloppings were monitored measuring pH and total titratable acidity (TTA) before and after fermentation. The pH was determined by a FiveEasy Plus pHmeter (Mettler-Toledo, Columbus, Ohio, USA) with a food penetration probe. TTA was defined as the amount of 0.1 M NaOH required to adjust the pH of 10 g dough in sterile water to 8.3.

2.3. Microbiological analysis and isolation of lactic acid bacteria and yeasts

Ten grams of sample (T0, T1 and T10) were suspended in 90 mL of sterile sodium chloride (0.9 %, *w/v*) solution and homogenized with a stomacher BagMixer 400 P (Interscience). Lactic acid bacteria were enumerated on modified MRS (mMRS) agar (Oxoid Ltd., Basingstoke, Hampshire, UK), supplemented with 0.01 % of cycloheximide (Sigma Chemical Co., USA), 1 % [wt/vol] maltose, 5 % [wt/vol] yeast extract (pH 5.6) at 30 °C for 48 h, under anaerobiosis. Yeasts and molds were cultivated on Sabouraud Dextrose Agar (SDA, Oxoid) supplemented with 0.01 % chloramphenicol at 25 °C for 48 h and 7 days respectively. Total mesophilic aerobic bacteria were enumerated on Plate Count Agar (Oxoid) supplemented with 0.01 % of cycloheximide under aerobic conditions at 30 °C for 48 h and *Enterobacteriaceae* were cultivated on Violet Red Bile Glucose Agar (Oxoid) at 37 °C for 48 h.

Ten-fifteen colonies of presumptive lactic acid bacteria, possibly with different morphology, were randomly taken from mMRS plates of T0, T1 and T10 at the highest dilutions and transferred to mMRS broth. LAB isolates were cultivated in mMRS at 30 °C for 24 h and re-streaked at least twice into the agar medium. LAB in T0 dough were isolated also upon enrichment. More specifically, 5 g of dough were added to 45 mL of mMRS supplemented with cycloheximide (0.01 %). After 24 h of incubation at 30 °C, 1 mL of enrichment broth was diluted with sterile sodium chloride solution and plated as described above.

Colonies of presumptive yeasts, possibly with different morphology, were also randomly taken from SDA plates of T0, T1 and T10 at the highest dilutions and transferred to Sabouraud broth. Catalase-positive yeast isolates were cultivated in Sabouraud at 25 °C for 24 h and re-streaked at least twice into the agar medium.

2.4. Genotypic characterization and identification of lactic acid bacteria

Bacteria genomic DNA was extracted using a Bacterial Genomic DNA Isolation Kit (Norgen Biotek Corp, Ontario, Canada) following the manufacturer's instructions. The obtained pure genomic DNA of isolates was stored at −20 °C for RAPD and 16S DNA sequencing analyses.

Three oligonucleotides, P7 5′-AGCAGCGTGG-3′, P4 5′-CCGCAGCGTT-3′, and M13 5′-GAGGGTGGCGGTTCT-3′, having arbitrarily sequences, were used for lactic acid bacteria isolates bio-typing. Reaction mixture and PCR conditions for primers were as described by Coda et al. (2006). Electrophoresis was carried out on agarose gel at 1.5 % (wt/vol) (Bio-Rad, Hercules, CA, USA) and the gel was visualized and imaged using GelDoc Go Gel Imaging System (Bio-Rad Laboratories GmbH, Munich, Germany). Molecular weight of the amplified DNA fragments was estimated by comparison with a molecular ruler (Bio-Rad) ranging from 100 to 1000 bp. The presence or absence of fragments was noted as 1 or 0, respectively. RAPD-PCR profiles were combined obtaining a dendrogram. Dice coefficients of similarity and UPGMA algorithm were used to estimate the similarity of the electrophoretic profiles. The primer pairs LpigF/LpigR, were used for amplifying the 16S rRNA gene fragment of lactic acid bacteria (De Angelis et al., 2006). Primers designed for the recA gene were also used to distinguish among *Lactiplantibacillus plantarum*, *Lactiplantibacillus pentosus*, *Lactiplantibacillus paraplantarum*, and *Lactiplantibacillus argentoratensis* species (Torriani et al., 2001; Bringel et al., 2005). PheS primers were used to identify at the species level within the genera *Leuconostoc* and *Weissella* (Naser et al., 2005). Electrophoresis was carried out on agarose gel at

1.5 % (wt/vol) (Bio-Rad) and before sequencing amplicons were purified with NucleoSpin® PCR & Gel Clean-up (Macherey-Nagel, Düren, Germany). Sequences alignments were carried out using the multiple sequence alignment method (Pontonio et al., 2015) and identification queries were fulfilled by a BLAST search in GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

2.5. Genotypic characterization and identification of yeasts

Yeasts genomic DNA was extracted using a Wizard® Genomic DNA Purification Kit (Promega Corp., Wisconsin, USA) following the manufacturer's instructions. To identify presumptive yeasts, two primers, NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′), were used to amplify the divergent D1-D2 domain of the 26S DNA (O'Donnell, 1993). Electrophoresis was carried out on agarose gel at 1.5 % (wt/vol) (Bio-Rad). Sequences alignments and identification of purified amplicons were carried out as described above.

2.6. Type II sourdoughs production, LAB, and yeasts pro-technological characterization

Type II sourdoughs (De Vuyst et al., 2023) were produced by using isolated LAB as starters, aiming at investigating their main pro-technological characteristics. The strains were cultivated in mMRS broth at 30 °C for 24 h. Cells were harvested by centrifugation (MicroCL-21R, Thermo Scientific) at 10,000 ×*g* for 10 min (4 °C), washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0), re-suspended in tap water and inoculated in sorghum doughs at the final cell density of log 7 cfu/g. Sorghum flour and tap water, containing the above cellular suspension of each LAB (cell density of ca. log10 7.0 cfu/g), were used to prepare 100 g of dough (DY 160). Doughs were fermented at 30 °C for 24 h. pH and TTA were monitored during fermentation as described above.

The kinetics of acidification were modelled according to the Gompertz equation as modified by Zwietering et al. (1990): $y = k + A \exp. \{-\exp[(Vmaxe/A)(\lambda - t) + 1]\}$; where y is the acidification extent expressed as ΔpH at the time t; k is the initial level of the depend variable to be modelled; A is the difference pH between inoculation and stationary phase; Vmax is the maximum acidification rate; λ is the length of the latency phase expressed in hours.

Water/salt-soluble extracts (WSE) of the sourdoughs were prepared (Weiss et al., 1993) and used to determine lactic and acetic acids, respectively with K-DLATE and K-ACET kits (Megazyme International Ireland Limited, Bray, Ireland). The fermentation quotient (FQ), defined as the molar ratio between lactic and acetic acids, was also determined. The quantification of free amino acids (FAA) was performed using a Biochrom 30+ series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, Cambridge, UK) coupled with a Li-cation-exchange column (4.6 × 200 mm internal diameter), as described by Verni et al. (2021).

Aiming at verifying their potential capability to synthesize EPS, the isolated LAB strains were inoculated on MRS agar (Oxoid) supplemented with 2 % sucrose (Sigma, Germany). EPS synthesis was observed through the examination of slimy colonies on the plate after 48 h of incubation at 30 °C.

Isolated yeasts cells, cultivated in Sabouraud at 25 °C for 24 h, were harvested by centrifugation as described above, re-suspended in tap water and used as starters for fermentation. Doughs, having DY 160 (cell density in the dough of ca. log 6.0 cfu/g), were produced with wheat flour. The dough leavening performance of the different yeasts was evaluated determining the volume increase of a 15 mL-dough placed in a graduated tube as described by Torreggiani et al. (2023). Results were expressed as difference between the initial and the final volume (ΔV, mL).

2.7. Untargeted metabolomic characterization of fermented sorghum

Five hundred milligrams of sorghum dough, before and after fermentation, were subjected to extraction, following a modified Bligh–Dyer protocol (Giampaoli et al., 2021). Briefly, each sample was ground in a mortar with liquid nitrogen and added to a cold mixture composed of chloroform (2 mL), methanol (2 mL), and water (1.4 mL). Samples were stirred, stored at 4 °C overnight, and centrifuged for 30 min at 11,000 ×g. The upper hydrophilic and the lower organic phases were carefully separated and dried under nitrogen flow. The dried residue of the hydrophilic phase was dissolved in 700 μL D2O solution of 3-(trimethylsilyl)- propionic-2,2,3,3,-d4 acid sodium salt (TSP, 2 mM) as an internal chemical shift and concentration standard. The hydrophilic phase was analyzed by ^{1}H NMR.

The NMR experiments were carried out at 298 K on a JNM-ECZ 600R (JEOL Ltd., Tokyo, Japan) spectrometer operating at the proton frequency of 600 MHz and equipped with a multinuclear z-gradient inverse probe head. The monodimensional ^{1}H NMR experiments were carried out for quantitative analysis, employing a presaturation pulse sequence for water suppression with a time length of 2 s, a spectral width of 9.03KHz and 64 k data points, corresponding to an acquisition time of 5.81 s. The pulse length of 90° flip angle was set to 8.3 μs, the recycle delay was set to 5.72 s. Monodimensional ^{1}H spectra were analyzed by ^{1}D NMR DELTA JEOL Ltd., Tokyo, Japan). Bidimensional ^{1}H—^{1}H TOCSY and ^{1}H—^{13}C HSQC experiments were acquired according to Spinelli et al. (2022) for the resonance assignment.

2.8. Analys of tannins

Tannins were analyzed through an HPLC-DAD method using epicatechin, epicatechin gallate, epigallocatechin gallate, and procyanidin B2 as standards. Two-hundred milligrams of samples were weighed and dissolved in 10 mL of Milli-Q water, then extracted at 90 °C for 2 h in a water bath. The samples were centrifuged at 4500 ×*g* for 10 min and the supernatant filtered through 0.22 μm filters for analysis (Kardel et al., 2013). HPLC analyses were performed with an HPLC system consisting of a 1260 Infinity II flexible pump, a 1260 Infinity II autosampler, and an HS 1260 Infinity II diode array detector (Agilent, Santa Clara, CA, USA). An InfinityLab Poroshell 120 EC-C18 column (3.0 mm × 150 mm, 2.7 μm) (Agilent, Santa Clara, CA, USA) was used as a stationary phase at 35 °C.

2.9. Statistical analysis

Sourdough propagation was carried out in duplicate, and each analysis was repeated in triplicate. Data from LAB and yeasts pro-technological characterization 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 Statistica 12.5 (StatSoft Inc., Tulsa, USA).

NMR data were statistically analyzed using MATLAB® R2023a (MathWorks, Natick, Massachusetts, USA) with the Statistics and Machine Learning Toolbox package was used as the program for univariate and multivariate analysis, with a home-built script. Univariate one-way ANOVA was performed. The Shapiro-Wilk test was performed on each variable to assess data normality prior to one-way ANOVA, while to verify the homoscedasticity condition, the Brown Forsythe (Brown and Forsythe, 1974) test was carried out, with a significance value of 0.05. If these conditions were not met, a non-parametric ANOVA test was carried out with Kruskal-Wallis (Stevens, 2013).

The data obtained from the chromatographic and spectroscopic characterization of sorghum type II sourdoughs were also analyzed using the MetaboAnalyst version 5.0 software (metaboanalyst.ca/; accessed online February 9th, 2024) to obtain a heatmap with hierarchical clustering (Ward's method) based on Euclidean distance. Correlations within the metabolic features ($p < 0.01$), assessed based on

Pearson's bivariate correlation, were determined with GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Sourdough fermentation

In the first 24 h of fermentation, sorghum dough pH decreased from 6.49 ± 0.04 (T0) to 5.51 ± 0.15, whereas TTA raised from 1.3 ± 0.1 to 4.1 ± 0.2 mL. During the backslopping, from the third day of propagation onward, the pH decreased below 4.5 and remained stable around 4.3 (mean value). Accordingly, TTA significantly ($P < 0.05$) increased compared to T0 and T1 (1.3 and 4.0 mL, respectively) fluctuating between 9.6 and 11.2 mL.

The sorghum dough (T0) was characterized by cell densities of 3.89 ± 0.27 and 1.2 ± 0.1 log cfu/g of presumptive LAB and yeasts, respectively, whereas total mesophilic aerobic bacteria and *Enterobacteriaceae* were 4.33 ± 0.12 and 3.75 ± 0.21 log cfu/g, respectively (Fig. 1). Molds were not detected at T0. After the first incubation at 30 °C (T1), cell density of both total mesophilic aerobic bacteria and presumptive LAB significantly ($P < 0.05$) increased to 6.63 ± 0.31 and 6.92 ± 0.24 log cfu/g, respectively, whereas yeasts and *Enterobacteriaceae* remained stable. After 10 days of propagation, followed by incubation at 30 °C for 8 h, a further increase was found for LAB and yeasts which reached 9.72 ± 0.68 and 6.91 ± 0.57 log cfu/g, while *Enterobacteriaceae* and molds were not found (Fig. 1).

3.2. Lactic acid bacteria and yeasts community

A total of 34 presumptive lactic acid bacteria were isolated from mMRS agar plates at the highest dilution and subjected to RAPD-PCR analysis and 16SrRNA sequencing (Fig. S1). Seventeen isolates were from sorghum dough (either with or without the enrichment step), nine and eight from spontaneously fermented doughs after 1 and 10 days of propagation, respectively. Gram-positive, catalase-negative, non-motile rods and cocci able to acidify the media were subjected to RAPD-PCR analysis. The 3 primers (M13, P4, and P7) employed for the analysis generated different profiles, having bands ranging from 2500 to 100 bp, which were used for clusters analysis. Comparing the PCR products obtained from three separate cultures of the same strain, the reproducibility of the RAPD fingerprints was assessed. At the similarity level of 80 %, the isolates were clustered in five groups (I-V, Fig. S1) except for T10I2, T10I3, A14, T1I1, T10I7, T10I7, T1I4, which were not grouped. Representatives of each cluster were identified by partial sequencing of the 16S rRNA and the following species were identified: *Lactiplantibacillus plantarum* (12 strains), *Weissella cibaria* (10), and *Weissella paramesenteroides* (12 strains) (Table S1). Most of the *W. paramesenteroides* were isolated after the enrichment procedures, whereas *L. plantarum* was found mainly when the sourdough was mature (T10).

After a preliminary morphological screening, 5 yeasts were isolated (from T1 and T10) and subjected to 26S DNA sequencing. The following species were identified (Table S1): *Saccharomyces cerevisiae* (4), *Meyerozyma guilliermondii* (1).

3.3. LAB and yeasts pro-technological performances

3.3.1. LAB kinetics of acidification and synthesis of organic acids

The 8 representative strains (*L. plantarum* T0I5, T10I1, T10I3, T10I5, AI4, *W. cibaria* T1I4, T1I8, *W. paramesenteroides* AI7) were singly used to ferment sorghum doughs at 30 °C for 24 h. Overall, all strains were able to acidify the doughs. *L. plantarum* strains showed the most intense acidification with a final pH of 3.85. Conversely, *L. plantarum* AI4 and *W. paramesenteroides* AI7 had the lowest ΔpH, 1.9 and 1.8, respectively (Fig. 2). Nevertheless, while for the parameters A (ΔpH) and Vmax (ΔpH/h) of the acidification kinetics emerged feeble differences among the strains, more heterogeneity was observed for the latency phase (λ). Indeed, *L. plantarum* T10I1 was the strains with the lowest λ, followed by *W. cibaria* T1I8, T1I4, and *L. plantarum* AI4 (1.64 ± 0.02, 1.94 ± 0.07; 2.22 ± 0.01, 2.29 ± 0.05 h, respectively).

Accordingly, lactic and acetic acid which were detected in traces before the incubation, increased in fermented doughs. Lactic acid concentration ranged from 23.8 to 25.1 mmol/kg and no significant differences ($P > 0.05$) were observed among the fermented doughs. On the contrary, depending on the starter used, relevant differences were found for the acetic acid production, which final concentration ranged from 5.48 to 20.5 mmol/kg, with *Weissella* strains being the highest producers (Table 1). Consequently, the FQ was lower for *W. paramesenteroides* AI7 (ca. 1.1), *W. cibaria* T1I4 and T1I8 (ca. 1.5) and higher for *L. plantarum* strains (from 2.4 to 4.3).

3.3.2. Effect of fermentation on proteolysis

The contribution of fermentation on the proteolysis was investigated through the quantification of FAA released in fermented sorghum doughs (Table 1). Before fermentation, total FAA were 774 ± 13 mg/kg of dough. In fermented sorghum, significant ($P < 0.05$) increases were observed. *L. plantarum* T0I5 and AI4 showed the lowest increases (17

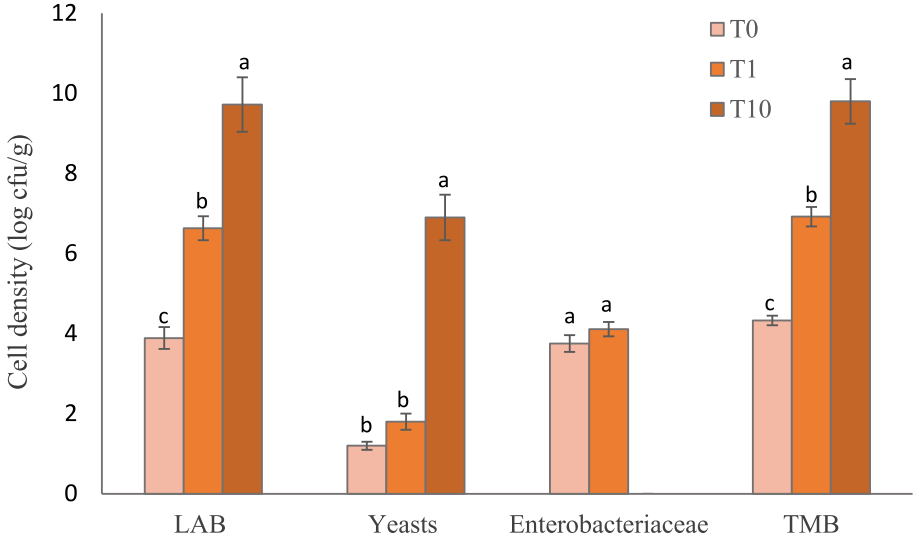

Fig. 1. Cell density, expressed as log cfu/g, of lactic acid bacteria (LAB), yeasts, *Enterobacteriaceae* and total mesophilic aerobic bacteria (TMB) of sorghum dough before (T0) and after 1 (T1) and 10 (T10) days of backslopping.

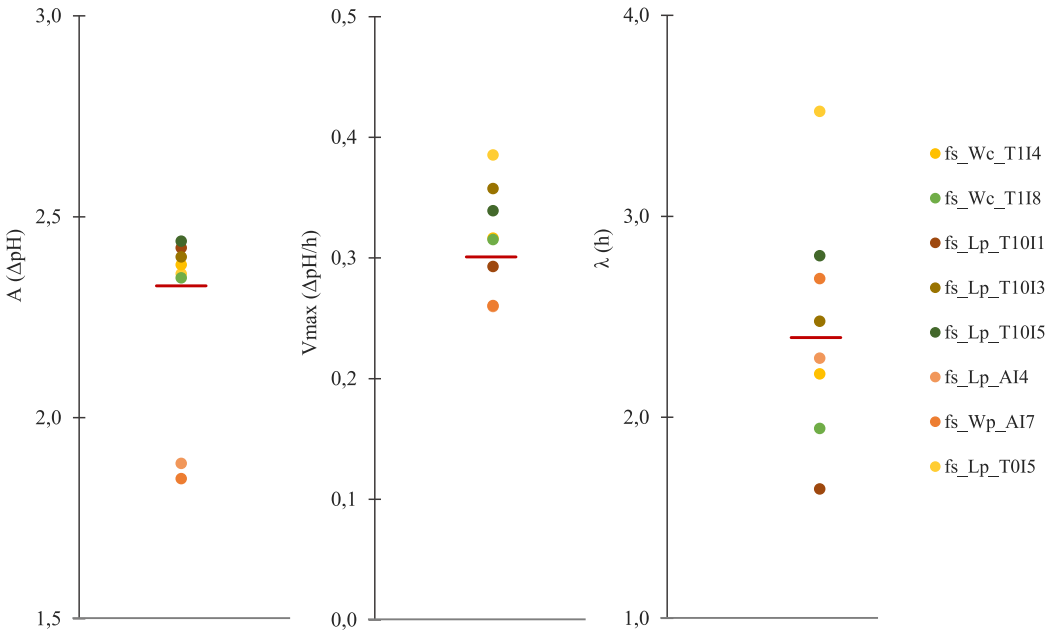

Fig. 2. Scatterplot based on the parameters of the acidification kinetics of LAB strains inoculated in sorghum flour doughs (cell density of ca. 7 log10 cfu/g) fermented at 30 °C for 24 h with *L. plantarum* T0I5 (fS_Lp_T0I5), *L. plantarum* T10I1 (fS_Lp_T10I1), *L. plantarum* T10I3 (fS_Lp_T10I3), *L. plantarum* T10I5 (fS_Lp_T10I5), *L. plantarum* AI4 (fS_Lp_AI4), *W. cibaria* T1I4 (fS_Wc_T1I4), *W. cibaria* T1I8 (fS_Wc_T1I8), and *W. paramesenteroides* AI7 (fS_Wp_AI7). Mean value is shown as a red line.

and 27 %, respectively). On the contrary, *W. cibaria* strains (T1I8 and T1I4) and *W. paramesenteroides* AI7 determined the highest increments of total FAA concentration reaching up to 1.7 g/kg. Indeed, except for Arg, the correlation matrix showed a strong positive relation (Fig. S2) between most amino acids, emphasizing the role of fermentation in the release of FAA.

Prior to fermentation, Asp, Asn, and Glu were the most abundant amino acids. After incubation, except for the doughs fermented with *W. cibaria* strains (T1I8 and T1I4) and *W. paramesenteroides* AI7, in which Asp remained the most abundant amino acid, Arg had the highest concentration. An inversely proportional trend to that observed for Arg, was found for Orn concentration, which significantly decreased ($P < 0.05$) in doughs fermented with *L. plantarum* strains (Table 1). Apart from *W. cibaria* T1I8, all the other LAB determined a decrease of Asn content from 15 to 60 % compared to prior fermentation (117 mg/kg). The most pronounced reduction was found in doughs fermented with *L. plantarum* strains (T0I5, T10I1, T10I5, and AI4). Overall, depending on the strain used, all fermented sorghum doughs had a GABA (γ-aminobutyric acid) content (mean value 104 mg/kg) at least 75 % higher compared to the control (Table 1).

3.3.3. EPS synthesis

A further characterization of the LAB strains, based on metabolic traits potentially relevant to the improvement of sorghum techno-functional properties, was carried out, assessing their ability to synthesize EPS. Under the conditions of this study, 3 out of 8 LAB strains showed the ability to produce EPS. Specifically, all the strains belonging to the *W. cibaria* and *W. paramesenteroides* species had a pronounced ability to produce EPS after 48 h incubation at 30 °C, whereas no production was observed for *L. plantarum* strains.

3.4. Yeasts leavening performances

The leavening performances of the isolated yeasts were evaluated measuring the volume increase of a wheat flour dough (Table 2), since the lack of gluten in sorghum flour would have hampered the results. Overall, in the first 2 h of fermentation, all the strains did not show an intense CO_2 production, especially *M. guilliermondii*. Among the isolates, *S. cerevisiae* T1S11 was the one that determined the highest ($P < 0.05$) volume increase. After 4 h of fermentation the volume exceeded 11 mL for all strains. The increment peak was reached after 6 h of leavening for *S. cerevisiae* T1S11 and T10S15 and *M. guilliermondii* T1S14, after 8 h for *S. cerevisiae* T1S10 and T10S14.

3.5. Type II sourdoughs metabolome profiling

A total of 34 metabolites were identified and quantified from ^{1}H NMR spectra of the aqueous extracts obtained from sorghum sourdoughs fermented with the 8 representative LAB strains in comparison to unfermented sorghum dough, used as control. A resonance assignment was carried out based on the signal chemical shift, multiplicity, TOCSY, HSQC and HMBC correlations. The ^{1}H chemical shifts, multiplicity and the ^{13}C chemical shifts of the identified molecules are reported in Table S2. This untargeted analysis, widely used to identify low molecular masses organic molecules in complex matrices, led to the identification of 29 different compounds in the control, while in sourdoughs, depending on the strain used, were found from 29 to 31 compounds. Overall, organic acids, carbohydrates, amino acids, as well as miscellaneous metabolites were detected.

Apart from the intense amino acids release characterizing sourdoughs obtained with *W. cibaria* strains, 1,2-propanediol and choline significantly increased from 1.62 ± 0.2 and 21.09 ± 2.97 mg/100 g (control) to 30.59 and 32.14 (mean values) mg/100 g, respectively, in the doughs fermented with *W. cibaria* T1I4 and T1I8. *W. cibaria* strains (T1I4 and T1I8) and *W. paramesenteroides* (AI7) led to the highest content of glutathione and dimethylamine (respectively up to 32 and 5.7 mg/100 g, compared to 0 and 0.40 of the control). On the contrary, *L. plantarum*-fermented doughs were characterized by higher content of 2-hydroxy-3-methylbutyric acid, hydroxybenzoate, ethanolamine, formic acid, and isopentanol. Compared to the control, although with small differences among the strains, all fermented doughs had higher content of allantoin and kynurenic acid. Whereas, guanine nucleotide, adenine nucleotide and fumaric acid disappeared after fermentation. As expected (Khoddami et al., 2023), HPLC-DAD confirmed tannins were at levels

Table 1
Metabolites concentration obtained from the chromatographic and spectroscopic characterization of raw (rS) and fermented (fS) sorghum. Fermentation was carried out for 24 h at 30 °C with *L. plantarum* T0I5 (fS_Lp_T0I5), *L. plantarum* T10I1 (fS_Lp_T10I1), *L. plantarum* T10I3 (fS_Lp_T10I3), *L. plantarum* T10I5 (fS_Lp_T10I5), *L. plantarum* AI4 (fS_Lp_AI4), *W. cibaria* T1I4 (fS_Wc_T1I4), *W. cibaria* T1I8 (fS_Wc_T1I8), and *W. paramesenteroides* AI7 (fS_Wp_AI7). Data are the means of three independent experiments ± standard deviations ($n = 3$). n.d., not detected.

	rS	fS_Lp_T0I5	fS_Lp_T10I1	fS_Lp_T10I3	fS_Lp_T10I5	fS_Lp_AI4	fS_Wc_T1I4	fS_Wc_T1I8	fS_Wp_AI7
Organic acids									
Lactic Acid (mmol/kg)	n.d.	25.06 ± 0.42^{a}	24.76 ± 0.45^{a}	23.55 ± 0.59^{a}	24.61 ± 0.34^{a}	23.47 ± 0.09^{a}	24.99 ± 0.12^{a}	24.45 ± 0.27^{a}	23.87 ± 0.15^{a}
Acetic Acid (mmol/kg)	0.37 ± 0.03^{f}	10.3 ± 0.20^{c}	7.39 ± 0.15^{d}	5.48 ± 0.02^{e}	7.86 ± 0.03^{d}	8.48 ± 0.02^{d}	16.65 ± 0.02^{b}	16 ± 0.01^{b}	20.05 ± 0.01^{a}
Formic Acid (mg/100 g)	0.39 ± 0.37^{b}	0.68 ± 0.1^{ab}	0.72 ± 0.09^{a}	0.81 ± 0.07^{a}	0.78 ± 0.11^{a}	0.73 ± 0.01^{a}	0.37 ± 0.27^{b}	0.62 ± 0.11^{ab}	0.43 ± 0.01^{b}
Citic Acid (mg/100 g)	46.19 ± 6.51^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	21.05 ± 2.78^{b}	16.83 ± 4.67^{b}	25.98 ± 4.13^{b}
Fumaric Acid (mg/100 g)	1.58 ± 0.3^{a}	0.06 ± 0.02^{b}	0.05 ± 0.04^{b}	0.07 ± 0.01^{b}	0.02 ± 0.04^{b}	n.d.	0.04 ± 0.03^{b}	0.05 ± 0.06^{b}	0.03 ± 0.03^{b}
Kynurenic acid (mg/100 g)	0.41 ± 0.2^{c}	1.37 ± 0.26^{ab}	1.96 ± 0.49^{a}	2.17 ± 0.28^{a}	2.05 ± 0.11^{a}	1.3 ± 0.09^{b}	2.15 ± 0.67^{a}	1.95 ± 0.36^{a}	1.73 ± 0.64^{a}
Hydroxybenzoate (mg/100 g)	1.17 ± 0.11^{b}	1.83 ± 0.15^{a}	2.01 ± 0.21^{a}	2.26 ± 0.25^{a}	1.98 ± 0.27^{a}	2.00 ± 0.07^{a}	n.d.	n.d.	n.d.
2-Hydroxy-3-methylbutyric acid (mg/100 g)	n.d.	1.66 ± 0.19^{a}	1.58 ± 0.25^{a}	1.85 ± 0.18^{a}	1.87 ± 0.21^{a}	1.72 ± 0.04^{a}	n.d.	n.d.	n.d.
Carbohydrates									
Glucose (mg/100 g)	358 ± 67^{b}	713 ± 79^{a}	886 ± 85^{a}	1111 ± 166^{a}	816 ± 91^{a}	949 ± 7^{a}	796 ± 31^{a}	944 ± 9^{a}	1066 ± 153^{a}
Galactose (mg/100 g)	6.77 ± 0.78^{d}	14.98 ± 0.87^{c}	19.08 ± 1.47^{b}	22.35 ± 1.29^{a}	17.47 ± 1.35^{bc}	19.87 ± 1.92^{b}	19.97 ± 1.74^{b}	28.88 ± 1.04^{a}	18.85 ± 1.26^{b}
Amino acids									
Asp (mg/kg)	42 ± 4^{d}	64.19 ± 2.04^{c}	70.12 ± 2.71^{bc}	75.33 ± 2.41^{b}	78.84 ± 2.5^{b}	72.51 ± 2.8^{b}	164 ± 5^{a}	183 ± 7^{a}	155 ± 5^{a}
Thr (mg/kg)	18.69 ± 0.6^{e}	26.39 ± 0.84^{d}	28.1 ± 1.08^{cd}	28.07 ± 0.9^{cd}	32.06 ± 1.02^{c}	27.29 ± 1.05^{cd}	40.94 ± 1.31^{ab}	47.16 ± 1.82^{a}	39.12 ± 1.24^{b}
Ser (mg/kg)	30.27 ± 0.97^{c}	9.96 ± 0.32^{f}	13.75 ± 0.53^{e}	11.77 ± 0.38^{ef}	13.36 ± 0.42^{e}	16.67 ± 0.64^{d}	67.57 ± 2.16^{b}	76.5 ± 2.95^{a}	65.14 ± 2.07^{b}
Asn (mg/kg)	118 ± 4^{a}	48.32 ± 1.53^{e}	60.52 ± 2.34^{d}	77.43 ± 2.47^{cd}	64.04 ± 2.03^{d}	49.17 ± 1.9^{e}	100 ± 3^{b}	116 ± 4^{a}	89.28 ± 2.83^{c}
Glu (mg/kg)	89.43 ± 2.86^{b}	49.07 ± 1.56^{d}	53.62 ± 2.07^{cd}	57.23 ± 1.83^{c}	60.6 ± 1.92^{c}	54.09 ± 2.09^{cd}	105 ± 3^{a}	113 ± 4^{a}	111 ± 3^{a}
Gly (mg/kg)	24.13 ± 0.77^{c}	37.01 ± 1.17^{b}	39.22 ± 1.51^{b}	39.31 ± 1.26^{b}	42.25 ± 1.34^{ab}	38.54 ± 1.49^{b}	43.41 ± 1.39^{a}	47.22 ± 1.82^{a}	42.5 ± 1.35^{ab}
Ala (mg/kg)	45.35 ± 1.45^{e}	62.45 ± 1.98^{d}	61.51 ± 2.37^{d}	65.26 ± 2.08^{d}	71.49 ± 2.27^{c}	59.49 ± 2.3^{d}	92.85 ± 2.97^{b}	108 ± 4^{a}	93.15 ± 2.96^{b}
Val (mg/kg)	20.96 ± 0.67^{e}	42.52 ± 1.35^{d}	49.26 ± 1.9^{c}	49.56 ± 1.58^{c}	51.14 ± 1.62^{c}	44.34 ± 1.71^{cd}	68.88 ± 2.2^{b}	78.93 ± 3.05^{a}	66.09 ± 2.1^{b}
Cys (mg/kg)	14.21 ± 0.45^{a}	2.59 ± 0.08^{c}	13.82 ± 0.53^{ab}	0.12 ± 0^{d}	11.95 ± 0.38^{b}	12.42 ± 0.48^{b}	14.77 ± 0.47^{a}	14.6 ± 0.56^{a}	13.63 ± 0.43^{a}
Met (mg/kg)	9 ± 0.29^{c}	18.8 ± 0.6^{b}	20.38 ± 0.79^{b}	22.03 ± 0.7^{b}	21.98 ± 0.7^{b}	19.49 ± 0.75^{b}	35.34 ± 1.13^{a}	39.43 ± 1.52^{a}	34.25 ± 1.09^{a}
Ile (mg/kg)	15.37 ± 0.49^{c}	21.1 ± 0.67^{b}	20.67 ± 0.8^{b}	22.82 ± 0.73^{b}	24.22 ± 0.77^{b}	21.53 ± 0.83^{b}	37.76 ± 1.21^{a}	42.7 ± 1.65^{a}	36.19 ± 1.15^{a}
Leu (mg/kg)	24.68 ± 0.79^{e}	85.37 ± 2.71^{d}	86.27 ± 3.33^{d}	97.95 ± 3.13^{c}	92.78 ± 2.94^{cd}	89.17 ± 3.44	116 ± 4^{ab}	124 ± 5^{a}	114 ± 4^{b}
Tyr (mg/kg)	19.18 ± 0.61^{c}	19.1 ± 0.61^{c}	21.28 ± 0.82^{c}	23 ± 0.73^{c}	22.23 ± 0.71^{c}	21.2 ± 0.82^{c}	49.63 ± 1.59^{a}	48.32 ± 1.86^{a}	41.88 ± 1.33^{b}
Phe (mg/kg)	15.75 ± 0.5^{d}	39.67 ± 1.26^{c}	43.08 ± 1.66^{c}	46.99 ± 1.5^{c}	43.24 ± 1.37^{c}	44.2 ± 1.71^{c}	67.8 ± 2.17^{ab}	72.35 ± 2.79^{a}	63.08 ± 2^{b}
GABA (mg/kg)	51.35 ± 1.64^{e}	89.81 ± 2.85^{d}	100 ± 4^{c}	116 ± 4^{b}	99.12 ± 3.14^{c}	90.91 ± 3.51^{d}	116 ± 4^{b}	130 ± 5^{a}	103 ± 3^{c}
Amm (mg/kg)	25.2 ± 0.81^{e}	28.99 ± 0.92^{d}	41.44 ± 1.6^{c}	35.89 ± 1.15^{cd}	40.65 ± 1.29^{c}	40.11 ± 1.55^{c}	92.59 ± 2.96^{b}	101 ± 4^{a}	91.84 ± 2.91^{b}
Orn (mg/kg)	1.18 ± 0.04^{d}	0.39 ± 0.01^{f}	0.38 ± 0.01^{f}	0.43 ± 0.01^{f}	0.71 ± 0.02^{e}	2.15 ± 0.08^{c}	66.46 ± 2.12^{b}	73.44 ± 2.83^{a}	65.3 ± 2.07^{b}
Lys (mg/kg)	26.14 ± 0.84^{e}	66.85 ± 2.12^{d}	73.73 ± 2.85^{c}	75.18 ± 2.4^{c}	76.62 ± 2.43^{c}	68.89 ± 2.66^{cd}	86.38 ± 2.76^{b}	96.49 ± 3.72^{a}	86.14 ± 2.73^{b}
His (mg/kg)	13.19 ± 0.42^{c}	17.18 ± 0.54^{b}	18.67 ± 0.72^{b}	19 ± 0.61^{b}	19.71 ± 0.63^{b}	17.44 ± 0.67^{b}	22.46 ± 0.72^{ab}	26.4 ± 1.02^{a}	23.18 ± 0.74^{ab}
Trp (mg/kg)	0.59 ± 0.02^{d}	12.95 ± 0.41^{b}	9.65 ± 0.37^{c}	10.59 ± 0.34^{c}	12.12 ± 0.38^{b}	13.28 ± 0.51^{ab}	12.05 ± 0.39^{b}	17.58 ± 0.68^{a}	14.7 ± 0.47^{ab}
Arg (mg/kg)	52.64 ± 1.68^{d}	123.91 ± 3.93^{b}	134 ± 5^{ab}	144 ± 5^{a}	134 ± 4^{ab}	129 ± 5^{b}	41.78 ± 1.33^{c}	53.93 ± 2.08^{c}	44.87 ± 1.42^{c}
Pro (mg/kg)	52.8 ± 1.69^{c}	54.66 ± 1.73^{bc}	63.11 ± 2.44^{b}	67.3 ± 2.15^{b}	62.31 ± 1.98^{b}	56.21 ± 2.17^{bc}	70.42 ± 2.25^{ab}	77.61 ± 3^{a}	67.35 ± 2.14^{b}

(*continued on next page*)

Table 1 (*continued*)

	rS	fS_Lp_T0I5	fS_Lp_T10I1	fS_Lp_T10I3	fS_Lp_T10I5	fS_Lp_AI4	fS_Wc_T1I4	fS_Wc_T1I8	fS_Wp_AI7
Miscellaneous metabolites									
Isopentanol (mg/100 g)	0.05 ± 0.01^{d}	0.66 ± 0.09^{a}	0.68 ± 0.13^{a}	0.87 ± 0.11^{a}	0.78 ± 0.16^{a}	0.66 ± 0.02^{ab}	0.44 ± 0.03^{c}	0.52 ± 0.06^{bc}	0.46 ± 0.06^{c}
1,2-propanediol (mg/100 g)	1.62 ± 0.2^{b}	1.41 ± 0.38^{b}	1.8 ± 0.43^{b}	2.1 ± 0.48^{b}	1.78 ± 0.27^{b}	1.65 ± 0.07^{b}	31.37 ± 17.81^{a}	29.81 ± 9.4^{a}	1.19 ± 0.18^{b}
Dimethylamine (mg/100 g)	0.4 ± 0.06^{d}	1.64 ± 0.43^{c}	3.85 ± 0.59^{b}	4.98 ± 0.08^{a}	1.27 ± 0.36^{c}	1.43 ± 0.14^{c}	5.33 ± 0.53^{a}	5.74 ± 1.05^{a}	4.64 ± 0.54^{a}
Etanolammine (mg/100 g)	2.25 ± 0.26^{b}	2.8 ± 0.4^{ab}	3.22 ± 0.47^{a}	3.59 ± 0.56^{a}	3.17 ± 0.47^{a}	3.35 ± 0.07^{a}	n.d.	n.d.	n.d.
Choline (mg/100 g)	21.09 ± 2.97^{b}	18.03 ± 3.06^{b}	21.06 ± 3.7^{b}	23.89 ± 4.38^{b}	21.25 ± 3.52^{b}	21.79 ± 0.07^{b}	30.12 ± 0.31^{a}	34.17 ± 3.12^{a}	29.53 ± 3.61^{a}
Allantoin (mg/100 g)	n.d.	4.12 ± 0.82^{ab}	4.73 ± 1.02^{ab}	6.01 ± 0.8^{a}	4.94 ± 1.4^{ab}	5.28 ± 0.07^{a}	4.99 ± 0.02^{b}	5.32 ± 0.45^{ab}	4.49 ± 0.81^{ab}
Glutatione (mg/100 g)	n.d.	11.77 ± 1.55^{b}	15.27 ± 2.18^{b}	14.61 ± 3.45^{b}	13.98 ± 1.05^{b}	14.61 ± 0.98^{b}	29.29 ± 2.18^{a}	31.24 ± 4.19^{a}	32.26 ± 3.88^{a}
Trigonelline (mg/100 g)	2.02 ± 0.13^{a}	1.2 ± 0.29^{ab}	1.49 ± 0.41^{ab}	1.9 ± 0.25^{ab}	1.43 ± 0.39^{ab}	1.56 ± 0.1^{b}	1.68 ± 0.11^{ab}	2.01 ± 0.33^{a}	1.54 ± 0.23^{ab}
Adenine (mg/100 g) Nucleotides	5.27 ± 2.07^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Guanine (mg/100 g) Nucleotides	1.55 ± 0.22^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Uracil (mg/100 g)	n.d.	0.72 ± 0.16^{ab}	0.8 ± 0.17^{ab}	1.04 ± 0.12^{ab}	0.76 ± 0.28^{ab}	0.7 ± 0.08^{b}	2.23 ± 0.64^{a}	2.8 ± 0.37^{a}	2.24 ± 0.39^{a}

The data are the means of three independent experiments ± standard deviations (n = 3).
[a-f]Different superscript letters indicate significant differences ($P < 0.05$).
Lactic and acetic acids, as well as some amino acids were identified by ^{1}H NMR analysis, but data reported in the table are the result of the analytical determinations reported in paragraph 2.6.

Table 2
Volume increase, ΔV(mL), of wheat flour dough leavened at 28 °C with the yeasts isolated from sorghum dough and sourdough.

	2 h	4 h	6 h	8 h
S. cerevisiae T1S11	5.54 ± 0.03^{Ca}	12.31 ± 0.23^{Ba}	14.16 ± 0.05^{Ab}	14.16 ± 0.10^{Ac}
S. cerevisiae T1S12	4.92 ± 0.09^{Da}	11.69 ± 0.14^{Cab}	16.00 ± 0.12^{Ba}	21.54 ± 0.16^{Aa}
M. guilliermondii T1S14	3.08 ± 0.18^{Cd}	11.08 ± 0.15^{Bb}	17.23 ± 0.04^{Aa}	17.85 ± 0.17^{Ab}
S. cerevisiae T10S14	4.31 ± 0.13^{Db}	11.02 ± 0.08^{Cb}	16.62 ± 0.11^{Ba}	21.40 ± 0.16^{Aa}
S. cerevisiae T10S15	3.69 ± 0.13^{Dc}	11.08 ± 0.09^{Cb}	15.39 ± 0.26^{ABab}	17.80 ± 0.13^{Ab}

The data are the means of three independent experiments ± standard deviations (n = 3).
Different uppercase letters indicate significant differences (P < 0.05) within the same sample among different incubation time. Different lowercase letters indicate significant differences (P < 0.05) among different sample within the same incubation time.

not detectable in the white sorghum flour used for the experiments.

4. Discussion

The widespread cultivation of sorghum and consequentially its implementation as part of the diet could highly affect food system sustainability, however, despite that, few aspects, among which the lack of gluten and the presence of antinutritional factors hinder its use as food ingredient. It is thus clear that green and efficient processing strategies are necessary to exploit its potential. Fermentation has been already reported as an effective technique to improve the nutritional, technological and sensory characteristics of sorghum flours (Rodríguez-España et al., 2022). Traditionally, during African cereals fermentations, which are mainly spontaneous and often contain sorghum, the succession of *Enterobacteriaceae*, followed by enterococci, lactococci, *Leuconostoc* and *Weissella* spp. is observed. At the end of fermentation, spontaneous fermented sourdoughs are characterized by the growth of pediococci, *L. plantarum* and *Limosilactobacillus fermentum* (Pswarayi and Gänzle, 2022). Nevertheless, to date, there are very little reports in the literature about the use of sorghum for a sourdough-type biotechnology, traditionally used for wheat sourdoughs, employing the backslopping procedure. Hence, the aim of this study was to investigate the role of lactic acid bacteria dominating sorghum sourdough and their impact on sorghum. The importance of isolating microorganisms from this type of sourdough relies on their ecophysiology. Indeed, sourdough ecosystem represents a stressful environment for residing microorganisms, hence allowing the growth and adaptation of a specific microbiota able to express the necessary physiological and stress responses for metabolic activities and survival (De Vuyst et al., 2023). Thus, the ad hoc selection of LAB and, eventually yeasts, dominating the sourdough and able to best adapt to such conditions, might offer competitive advantages in modulating the fermentation process towards specific goals, when used as starters.

Type I sourdoughs typically have low DY (⟨200) and are produced at temperatures between 20 and 30 °C with a short to moderate fermentation time (6–24 h) necessary to reach a pH of ca. 4.0. The daily refreshments keep LAB and yeasts in sourdough metabolically active (De Vuyst et al., 2023). Accordingly, a traditional sourdough, obtained through a backslopping procedure in which sorghum dough (DY 160) was spontaneously fermented (at 30 °C) and then used as active starter culture for the inoculum for a new dough, was used in the first part of the study. In type I sourdoughs, fermentation starts with autochthonous bacteria, among which proteobacteria, staphylococci and several LAB as well as yeasts species. The growth of autochthonous LAB species causes a fast acidification followed by the stabilization of sourdough-specific LAB species that prevail in the mature sourdoughs (De Vuyst et al., 2023). As confirmed by the microbiological and biochemical analysis, in the first few days of refreshments pH reduction was minimal, and the dough was characterized by higher densities of *Enterobacteriaceae* (Fig. 1). Nevertheless, as the pH dropped (from T3 onward), *Enterobacteriaceae* decreased whereas LAB and yeasts dominated the sourdough.

Lactic acid bacteria dominating sorghum flour and sourdough were thus identified by culture-dependent analysis revealing *L. plantarum* as the predominant species found in the mature sourdough, whereas *W. cibaria* and *W. paramesenteroides* were the species isolated the most

from the MRS-enriched flour and after the first refreshment. These results are consistent with those found by Falasconi et al. (2020), who isolated *Weissella* species (*W. cibaria, W. confusa* and *W. paramesenteroides*), in sorghum sourdough only in the early stages of backslopping (up until the first day of refreshments). Similarly, the occurrence of *L. plantarum* and *Weissella* spp. in spontaneously fermented sorghum was previously reported (Madoroba et al., 2011; Pswarayi and Gänzle, 2022). On the other hand, the environmental pressure exerted by sorghum phenolic compounds is the likely cause of the dominance of *L. plantarum* strains in sorghum flour and sourdough. Indeed, while in cereals like wheat or rye, the concentration of phenolic compounds is far from their inhibitory concentration, in sorghum it is higher than the inhibitory concentration for sensitive lactobacilli (Gänzle, 2014). Hence, it is suggested that the presence of phenolic compounds with antimicrobial activity in sorghum selects a microbiota resistant to phenolic compounds and species like *Fructilactobacillus sanfranciscensis*, commonly present in sourdoughs obtained with other cereals, are inhibited by polyphenols, thus failing to grow in sorghum sourdough (De Vuyst et al., 2023; Gänzle, 2014). Having antimicrobial activity, phenolic compounds select for *L. plantarum*, *Lm. fermentum* and *Furfurilactobacillus milii* that are more resistant to sorghum phenolic compounds, converting them into compounds with lower antimicrobial activity (Gaur et al., 2023). On the contrary, *L. plantarum* is the species most frequently found in fermented plant-based foods where phenolic compounds are abundant, hereby explaining why it was the dominant species in the sorghum sourdough produced in this study.

For the identification of yeast isolates, 26S rRNA gene sequence analysis revealed a prevalence of *S. cerevisiae* (4 out of 5 isolates), nevertheless, at T1 *M. guilliermondii* was detected. Although *S. cerevisiae* and *Candida humilis* are among the predominant yeast species found in sourdough, *M. guilliermondii* is occasionally isolated from wheat sourdough (De Vuyst et al., 2016). To the best of our knowledges there are no reports in the literature focused on yeast species isolated from sorghum type I sourdough, however, Ogunsakin et al. (2017) isolated *S. cerevisiae* from spontaneous fermented (48 h) sorghum. *M. guilliermondii*, instead, was found in a sorghum beer and considered as a spoilage microorganism, due to the high concentrations of acetaldehyde produced and its negative impact on beer flavor (Attchelouwa et al., 2018; Attchelouwa et al., 2022). Nevertheless, since the isolated yeasts did not exhibit extensive leavening potential, for instance compared to commercial baker's yeast (data not showed), the study focused on the characterization of LAB pro-technological and functional performances as starter.

Representative LAB strains of the sorghum sourdough microbiota (*L. plantarum* T0I5, T10I1, T10I3, T10I5, AI4, *W. cibaria* T1I4, T1I8, *W. paramesenteroides* AI7) were thus selected as starters to produce type II sourdoughs, which are characterized by a starter culture-initiated fermentation process (De Vuyst et al., 2023). All the strains were able to grow and acidify sorghum dough producing lactic and acetic acids. Nevertheless, *L. plantarum* T10I1 and *W. cibaria* T1I8 and T1I4 showed the best adaptation to the matrix. Acidification is a direct consequence of carbohydrate metabolism, and, although slight differences were observed among strains, this phenomenon was already observed during sorghum fermentation (Galle et al., 2010) and ascribed to the activity of endogenous glucoamylases, which, unlike amylases, are not inhibited at pH lower than 4.5 (Gänzle, 2014). Moreover, since maltose is scarcely present in sorghum flour, compared to wheat, and glucose is the preferred source of carbon in *W. cibaria* (Galle et al., 2010; Paramithiotis et al., 2007), this also explains the widespread occurrence of *Weissella* spp. rather than *F. sanfranciscensis* which prefers maltose. Being obligately heterofermentative LAB (Bello et al., 2022), *Weissella* strains produced the highest quantities of acetic acid and were the only strains able to synthesize exopolysaccharides from sucrose. EPS from the *Weissella* genus have several techno-functional and biological properties; they are known for their high water-holding capacity, which makes them highly appealing to improve the structure of gluten free products, and they can have antioxidant, antimicrobial, immunomodulatory activities, as well as prebiotic potential (Kavitake et al., 2020). In *Weissella cibaria*-fermented sorghum doughs higher concentration of 1,2-propanediol, which is a metabolite of lactic acid, were found. 1,2-propanediol was found in sorghum fermented by *Lentilactobacillus parabuchneri* (Sekwati-Monang et al., 2012), however, to date there are no reports of *Weissella* species encoding for the genes responsible of such conversion. Hence it is unclear whether 1,2-propanediol is the result of the strain metabolic activity or that of sorghum autochthonous microbiota.

The permutation analysis (Fig. 3), based on the metabolome characterization, clearly distinguished raw and fermented sorghum doughs. Raw sorghum was characterized by lower content of almost all the compounds identified with the NMR analysis as well as FAA. On the other hand, the metabolic profile of the strains mainly grouped together *W. cibaria* strains (T1I4 and T1I8) and *W. paramesenteroides* AI7 which distinguished for the high amino acids content released but also for the presence of compounds, particularly interesting from a physiological perspective, whose concentration increased during fermentation in a species or strain specific matter (Fig. 3). The intense proteolysis, as abundantly studied in sourdoughs (Gänzle and Gobbetti, 2023), is explained by a combination of factors, *i*) the presence of proteases endogenous of sorghum flour, *ii*) the acidification occurred during fermentation, which activates proteases that have optimum pH below 4.5, and *iii*) the presence of peptidases, and amino acid/peptide transporters of lactic acid bacteria. It is interesting to notice how, whereas in all fermented samples most FAA increased, in those fermented with *L. plantarum* strains asparagine decreased. Asn is one of the compounds that once condensed with a carbonyl source in heated foods form acrylamide a toxic substance that has become a health issue due to its carcinogenic potential (Hamzalıoğlu and Gökmen, 2024). One of the strategies used to decrease acrylamide content in baked goods is to use asparaginase which can be added to the dough directly or indirectly using microorganisms. Indeed, a decrease of acrylamide formation in rye crispbread due to sourdough fermentations was recently reported by Ameur et al. (2024). Hence, this trait could be further studied in sorghum isolates. In this case, since the concentration of aspartate and ammonium, products of Asn hydrolysis by asparaginase, was higher in *L. plantarum* fermented doughs compared to the control, the decrease of Asn was ascribed to the metabolism of *L. plantarum* which possess asparaginases (Mathiyalagan et al., 2021).

A negative correlation ($P < 0.01$, Fig. S2) was found for Arg and Orn, thus hypothesizing the involvement of the arginine-deaminase (ADI) pathway, that catalyzes the conversion of arginine to ornithine, citrulline, ammonia, and carbon dioxide, while generating ATP (Gänzle et al., 2007). Sorghum fermented with *W. cibaria* strains (T1I4 and T1I8) and *W. paramesenteroides* AI7 were the once with the lowest Arg content, and although the distribution of genes associated with the ADI pathway varies among bacteria, *Weissella* species were found to exhibit high arginine catabolic activity (Jung et al., 2021), thus explaining the lower Arg content in *Weissella*-fermented sorghum compared to those fermented with *L. plantarum* strains. 2-Hydroxy-3-methylbutyric acid, which was abundant in sorghum fermented with *L. plantarum* strains, is a valine derivative that was found to promote intestinal epithelial cells proliferation, potentially improving the gut barrier function (Qiao et al., 2022). Trp, instead, is metabolized through two major pathways, the kynurenine pathway and the methoxyindole pathway (Tsuji et al., 2023). While the latter generates serotonin, the former has as product kynurenic acid, which was from 3- to 5-fold higher in fermented sorghum compared the control dough. Kynurenic acid has neuroprotective properties and is able to reduce pro-inflammatory cytokines (Tsuji et al., 2023). GABA is among the other amino acid derivatives that increased during fermentation. Produced by LAB glutamic acid decarboxylase as a response to acid stress, GABA is a neurotransmitter with numerous physiological functions, including hypotensive activity, diuretic, tranquilizing, analgesic, and anti-diabetic effects (Pannerchelvan et al., 2023). GABA content ranged from 89 to 130 mg/kg, which can be

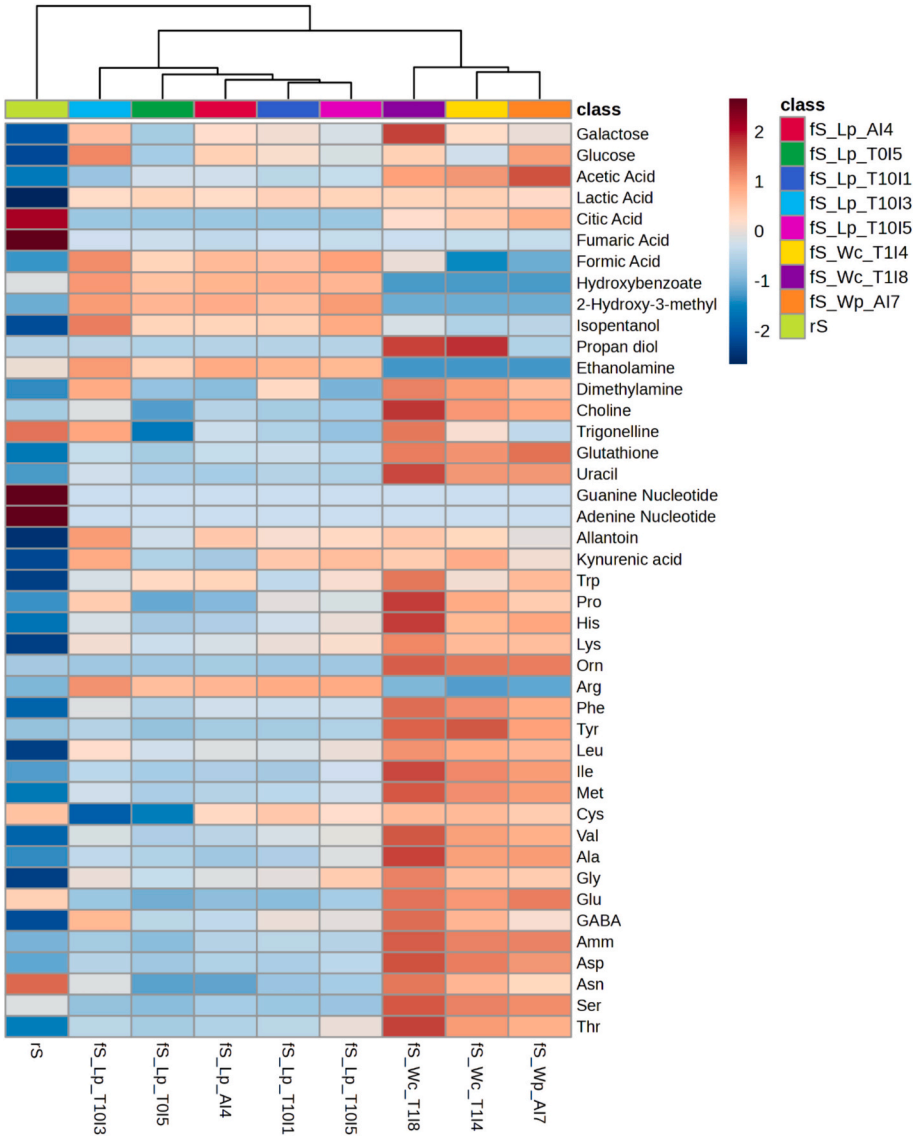

Fig. 3. Hierarchical clustering heatmap of metabolites concentration obtained from the chromatographic and spectroscopic characterization of raw (rS) and fermented (fS) sorghum. Fermentation was carried out for 24 h at 30 °C with *L. plantarum* T0I5 (fS_Lp_T0I5), *L. plantarum* T10I1 (fS_Lp_T10I1), *L. plantarum* T10I3 (fS_Lp_T10I3), *L. plantarum* T10I5 (fS_Lp_T10I5), *L. plantarum* AI4 (fS_Lp_AI4), *W. cibaria* T1I4 (fS_Wc_T1I4), *W. cibaria* T1I8 (fS_Wc_T1I8), and *W. paramesenteroides* AI7 (fS_Wp_AI7). The color scale-bar ranged between low (blue) to high (brown) concentrations that were used to perform the clustering (Ward's method) based on Euclidean distance.

considered a discrete production for sorghum. Indeed, in a previous paper, fermentation of two sorghum varieties (red and white) with LAB strains yielded up to 45 mg/kg of GABA (Garzón et al., 2020), less than half of the amount obtained in our work. Moreover, studies reported antihypertensive effects with daily consumption of 10 mg or 16.8 mg of GABA, as well as with a single oral administration of 0.5 mg per kg body weight (for a review see Rashmi et al., 2018). Hence the GABA obtained in this study could be enough to provide a biological activity, yet, of course, high GABA-producing strains could be selected if enriching sorghum in even higher content of GABA were to be the goal.

Glutathione and allantoin are among the compounds which significantly increased after fermentation and could potentially increase sorghum functionality. Glutathione (GSH), a low molecular weight thiol, is a tripeptide (Glu-Cys-Gly), known for its ability to maintain the intracellular redox homeostasis and protect cells against oxidative damage, yet also has several roles in biological systems, including immune boosting and cellular detoxifying activities (Pophaly et al., 2012). GSH has been reported to protect Gram negative bacteria cells from acid stress by regulating K^+ transport and thus maintaining the cytoplasmic pH, but also acts as sulfur and nutrient source, thus promoting the growth of several LAB (Pophaly et al., 2012). Species of the former *Lactobacillus* genus, including *L. plantarum*, were found to be negative for GSH de novo synthesis (Pophaly et al., 2017), however, glutathione reductase activity of sourdough lactobacilli increases thiol levels in sourdough (Vermeulen et al., 2006). Glutathione reductase activity was also found in *Weissella* species (Martínez et al., 2020). Moreover, in sorghum, and plants in general, glutathione exists in two forms, reduced and oxidized, and changes in this ratio mainly depends on pH (Malmir, 2011); this would suggest that the increase found after fermentation (on average, 14 and 30 times higher in doughs fermented with *L. plantarum* and *W. cibaria*, compared to sorghum flour) is likely due to a combination of the acidification as well as LAB glutathione reductase activity. This hypothesis could be corroborated by the strong positive correlation between acetic acid and glutathione ($P < 0.01$, Fig. S2). Ubiquitous of the plant kingdom due to its involvement in plants stress tolerance, allantoin is an intermediate product of purine catabolic pathway (Kaur

et al., 2021). Allantoin is known for its antidiabetic effects due to its ability to decrease plasma glucose levels (Niu et al., 2010), it is approved by the Food and Drug Administration in skin creams for skin soothing, healing, anti-irritating, and protection effects. Allantoin was not found in the control, whereas ca. 5 mg/100 g were found in fermented samples, although without significative differences ($P < 0.05$) among the strains. Allantoin can be produced by some LAB including *L. plantarum*, from uric acid through uricase in presence of oxygen (Handayani et al., 2018). It is possible that the reaction happened during the first few hours of fermentation, when there was still oxygen in the headspace of the container were fermentation occurred, before anaerobic conditions were established. It was shown that the administration of probiotics, among which *L. plantarum* strains, induced changes in rats fecal metabolite, increasing allantoin concentration, thus leading to the suppression of cholesterol biosynthesis (Yap et al., 2020). Nevertheless, it should be noted that although uricase activity was studied in lactic acid bacteria (Handayani et al., 2018; Iswantini et al., 2009), to date, the presence of the gene encoding for uricase has never been demonstrated, hence the contribution of the endogenous microbiota cannot be entirely excluded.

Overall, all the isolated strains showed a good potential to be used as starters for sorghum fermentation. While *Weissella* strains distinguished for the amino acid catabolism, especially *W. cibaria* T1I4 and T1I8, and the synthesis of techno-functional compounds like EPS, *L. plantarum* strains asparaginase activity was particularly efficient, as it was their ability to increase allantoin and kynurenic acid content. Further research might focus on the study of their capability fully enhance these aspects when used as a mixed culture starter. The investigation of sourdough fermentation in sorghum utilization presents an opportunity to enhance the nutritional and functional value of such important cereal crop. Thus, understanding the intricate relationship between microbial ecology, nutrient enhancement, and the broader implications for food security is crucial for maximizing the potential of fermentation in promoting a more sustainable food system.

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CRediT authorship contribution statement

Michela Verni: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Andrea Torreggiani:** Formal analysis, Data curation. **Adriano Patriarca:** Formal analysis, Data curation. **Elisa Brasili:** Resources, Methodology, Investigation. **Fabio Sciubba:** Resources, Methodology, Investigation. **Carlo Giuseppe Rizzello:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Carlo Giuseppe Rizzello reports financial support was provided by European Union Next-GenerationEU. If there are other authors, they 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

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2024.110805.

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