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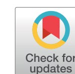
Exploitation of autochthonous Tuscan sourdough yeasts as potential starters



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ABSTRACT

The increasing demand for healthy baked goods boosted studies on sourdough microbiota with beneficial metabolic traits, to be used as potential functional starters. Here, yeast populations of traditional sourdoughs collected from four Tuscan bakeries were investigated. Among 200 isolated strains, 78 were randomly selected and molecularly characterized. *Saccharomyces cerevisiae* was dominant, representing the only species detected in three out of the four sourdoughs. The fourth one harbored also *Kazachstania humilis*. Inter-delta regions analysis revealed a high intraspecific polymorphism discriminating 16 biotypes of *S. cerevisiae* isolates, which clustered based on their origin. Representative isolates from each biotype group were individually used to ferment soft and durum wheat flour, aiming at evaluating their pro-technological, nutritional and functional features. During fermentation under standardized conditions, all strains were able to grow of ca. 2 log cycles, but only *S. cerevisiae* L10Y, D18Y and D20Y had a significantly shorter latency phase in both flours. Overall, the highest volumes were reached after 16 h of fermentation in both soft and durum fermented dough. *S. cerevisiae* D2Y produced the highest dough volume increase. *K. humilis* G23Y was the only strain able to increase the total free amino acids concentration of the doughs. Overall, values of phytase activity were significantly higher in durum compared to the corresponding soft fermented dough. *K. humilis* G23Y and *S. cerevisiae* D20Y, D24Y showed a threefold higher phytase activity than spontaneously fermented control, and the highest concentration of total phenols. Almost all the strains led to increases of antioxidant activity, without significant differences among them. Investigations on the resistance of the strains to simulated gastric and intestinal conditions, that is considered a pre-requisite for the selection of probiotics, revealed the ability to survive in vitro by many of the strains considered. This study proposed the best performing yeast strains selected among autochthonous sourdough yeasts based on their pro-technological, nutritional and functional traits to be used as starters for making sourdough baked goods or functional cereal-based beverages. Although some yeast strains combined several technological and nutritional traits, the association of more selected strains seemed to be a requisite to get optimal sourdough characteristics.

1. Introduction

In the last decade food production and consumption have undergone rapid changes, related to an increasing demand for natural and healthy foods. Such trend involved also the cereal-based food and beverages. Cereals are considered one of the most important sources of dietary carbohydrates, proteins, vitamins, minerals and fibers for people all over the world (Blandino et al., 2003). Nowadays, cereals alone or mixed with other ingredients are used for the production of traditional fermented beverages as well as for the development of new foods with enhanced healthy properties (Coda et al., 2012).

Sourdough fermentation represents one of the oldest food biotechnologies to ferment cereal matrices, which was mainly studied for its effect on the sensory, nutritional, functional, structural and shelf life properties of leavened baked goods (Gobbetti et al., 2014). A microbial consortium mainly consisting of obligately and/or facultatively heterofermentative lactobacilli and yeasts usually dominates the mature sourdough (Vogel et al., 1996). Usually, the diversity of lactic acid bacteria is larger than that of the yeast microbiota, since *Saccharomyces cerevisiae* very frequently was the only dominating species in sourdough

Abbreviations: ME, Methanolic Extract; TFAA, Total Free Amino Acids; TTA, Total Titratable Acidity; DY, Dough Yield

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(De Vuyst et al., 2009). Yeasts are primarily responsible for the leavening, while Lactic Acid Bacteria (LAB) for the acidification of the dough, and both contribute to the flavour of the resulting breads (Valmorri et al., 2010). Although almost all the functional and nutritional features that are attributed to sourdough are mainly the consequence of the metabolism of LAB (Gobbetti et al., 2014; Rizzello et al., 2016), an increasing number of studies highlighted that also yeasts may affect such features (De Vuyst et al., 2009, 2016; Katina and Poutanen, 2013; Perricone et al., 2014). Indeed, several yeast strains exert several functional properties, such as vitamin production (Kariluoto et al., 2014; Liukkonen et al., 2003), phytase (Türk et al., 2000) and antioxidant (Moore et al., 2007; Wang et al., 2014) activities as well probiotic properties (Moslehi-Jenabian et al., 2010).

Worldwide, *S. cerevisiae* is the species most frequently retrieved from sourdough and other fermented foods. The other sourdough yeasts belong to the species *Kazachstania humilis*, *Wickerhamomyces anomalus*, *Torulaspora delbrueckii*, *Kazachstania exigua*, *Pichia kudriavzevii* and *Candida glabrata*. Although single stable sourdoughs may harbor only one or two species (De Vuyst et al., 2016), a large intraspecific genetic diversity has been reported by several authors (Huys et al., 2013), mirrored by a wide functional variability. As functional traits are strictly strain-dependent, the characterization of sourdough yeast strains is crucial for their biotechnological use in order to obtain functional cereal-based foods and beverages. Already strains of *S. cerevisiae* are commercially used as probiotics. Although sourdough-associated yeasts could also offer probiotic advantages.

Sourdough is a stressful environment, characterized by a low pH, low oxygen tension, temperature fluctuation and by the presence of carbohydrates. Additionally, native yeast microbiota is well adapted to such ecosystem and process (De Vuyst et al., 2016). For this reason, suitable yeast starter strains for bread making or cereal-based beverages are often selected among sourdough autochthonous isolates, in order to guarantee their stable persistence. So far, such strains have been selected for their peculiar leavening and technological properties (Guerzoni et al., 2013), without taking into account their functional characteristics and ability to enhance the nutritional and nutraceutical properties of the derived products. Here, we isolated and molecularly characterized autochthonous yeast strains from Tuscan sourdoughs and investigated their pro-technological and functional traits to be used as potential functional starters for biotechnological applications.

2. Materials and methods

2.1. Microbiological characterization of four Tuscan sourdoughs

The sourdoughs analysed in this study were collected from different Tuscan bakeries located in the provinces of Pistoia and Livorno, here named C, D, G and L, which produced sourdough bread without any added salt. Ten g of sourdough were homogenized with 90 mL of saline-peptone water (9 g/L NaCl, 1 g/L bacteriological peptone, Oxoid, Milan, Italy) in a sterile stomacher bag for 2 min at 260 rpm, using a Stomacher (Stomacher 400, Laboratory Blender). Yeast numbers were determined using Wallerstein Laboratory Nutrient (WLN) agar (Oxoid, Basingstoke, UK) and Yeast Extract Peptone Dextrose (YEPD) agar. To inhibit bacterial grow, both media were supplemented with 100 mg/L chloramphenicol and incubated at 28 °C for 48 h. Analyses were carried out in triplicate.

At least 15 colonies of yeasts were randomly selected from each WLN plate, based on phenotypic colony characteristics, and then purified by streaking four times onto the same medium.

Each strain was named with the acronym of the collection of the Microbiology Laboratories of the Department of Agriculture, Food and Environment of the University of Pisa (IMA, International Microbial Archives), followed by the name of the bakery, a progressive number, plus "Y". Purified strains were stored at −80 °C in the same broth medium used for isolation, supplemented with 20% (*w/v*) glycerol.

Table 1
Yeast collection strains and commercial baker's yeasts used in this study.

Strains[a]	Source of isolation
Kazachstania humilis DBVPG 6753[T]	San Francisco sourdough
Kazachstania humilis DBVPG 7219[T]	Bantu beer
Kazachstania exigua DBVPG 6956	Wheat sourdough, Italy
Saccharomyces cerevisiae ATCC 32167	Unknow
Saccharomyces cerevisiae IMA 19Y	PDO Tuscan bread sourdough
Saccharomyces cerevisiae IMA 36Y	PDO Tuscan bread sourdough
Saccharomyces cerevisiae IMA 105Y	PDO Tuscan bread sourdough
Saccharomyces cerevisiae Zeus (Zeus IBA)	Unknow
Saccharomyces cerevisiae Lievitalia (Lesaffre Italia)	Unknow

[T] Type Strain.
[a] DBVPG = International Collection of Department of Agricultural, Food and Environmental Science, University of Perugia, Perugia, Italy; ATCC = American Type culture Collection, Manassas, Virginia, USA; IMA = International Microbial Archives, Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy.

2.2. Genotypic identification of isolates

Genomic DNA from pure cultures of yeasts both isolated from sourdoughs and belonging to International Culture collections (Table 1), was extracted using "MasterPure™ Yeast DNA Purification Kit" (Epicentre®), according to the manufacturer's instructions. Yeast strains were identified by 5.8S-ITS regions amplification and their Restriction Fragment Length Polymorphism (RFLP) analysis. The amplification was performed using ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) primer (White et al., 1990) as reported in Palla et al. (2017). Amplicons were digested at 37 °C overnight with the restriction endonucleases *Hae*III and *Hpa*II (BioLabs, Ipswich, MA, USA). The restriction fragments were separated on 2% (*w/v*) agarose gels stained with ethidium bromide (0.5 μg/mL) in Tris-borate-EDTA buffer (Sigma-Aldrich, Milan, Italy) at 70 V for 1 h. A 100 bp DNA ladder (BioLabs) was used as a molecular weight marker. All gels were visualized by UV and captured as TIFF format files by the UVI 1D v. 16.11a program for FIRE READER V4 gel documentation systems (Uvitec Cambridge, Eppendorf).

2.3. Molecular characterization at strain level

In order to discriminate the yeast isolates at strain level, a preliminary identification of the best performing molecular technique was carried out on five isolates, using the following fingerprinting techniques: Restriction Fragment Length Polymorphism analysis of mitochondrial DNA (RFLP mtDNA) (Agnolucci et al., 2007, 2009), mitochondrial COX1 gene introns amplification (Lopez et al., 2003), repetitive sequence Polymerase Chain Reaction (Lattanzi et al., 2013), Random Amplified Polymorphic DNA (RAPD) (Succi et al., 2003) and inter-delta regions amplification (Ness et al., 1993).

The intraspecific diversity of the 78 yeast isolates, along with the collection strains and two commercial baker's yeasts (Table 1), was carried out by inter-delta regions analysis, as it resulted the best performing technique. Amplification reaction was performed in a final volume of 50 μL, containing 5 μL of 10× DyNAzyme Buffer Mg^{2+}-free (Finnzymes, Thermofisher, Milan, Italy), 4 mM $MgCl_2$, 0.2 mM of each dNTP (EuroClone), 5 μM of δ_1 (5′-CAAAATTCACCTATA/TTCTCA-3′) and δ_2 (5′-GTGGATTTTTATTCCAACA-3′) (Ness et al., 1993) primer (Eurofins Genomics, Ebersberg Germany), 1.25 U of Taq DyNAzyme II DNA polymerase (Finnzymes) and 160 ng of DNA. PCR amplification was carried out with an iCycler-iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Milan, Italy), using the following conditions: 97 °C initial denaturation for 4 min; 30 amplification cycles of 30 s at 94 °C, 1 min at 45 °C, 2 min at 72 °C; final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on 1.8% (*w/v*) agarose gel

stained with ethidium bromide (0.5 μg/mL) at 80 V for 1 h. All gels were visualized by UV and captured as TIFF format files by the UVI 1D v. 16.11a program for FIRE READER V4 gel documentation systems (Uvitec Cambridge, Eppendorf). Inter-delta profiles were digitally processed and analysed with BioNumerics software version 7.6 (Applied Maths, St-Martens-Latem, Belgium). Profiles were compared using the band matching tool with a position tolerance and optimization of 0.5% and similarity was calculated using Dice's coefficient. For cluster analysis, Unweighted Pair Group Method with Arithmetic means (UPGMA) trees with highest resampling support, in a permutation sample of size 200, were constructed. The reproducibility of fingerprints was assessed on four randomly selected strains.

2.4. Pro-technological properties of selected strains

Selected yeast strains were cultivated into Sabouraud Dextrose Broth (SDB, Oxoid, Basingstoke, Hampshire, UK) at 25 °C for 48 h. Aiming at investigating the main pro-technological and functional characteristics, cells were harvested by centrifugation (10,000 × *g*, 10 min, 4 °C), washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0), re-suspended in tap water at the cell density of ca. 6.0–7.0 Log cfu/mL and used as starters for soft (*Triticum aestivum*) and durum (*Triticum turgidum* ssp. *durum*) wheat flours dough fermentation. The proximate composition of soft and durum flours was as follows: moisture, 13.5 ± 0.67% and 15.5 ± 0.21%; protein (N × 5.7), 12.5 ± 0.01%, and 9 ± 0.02% of dry matter (d.m.); total carbohydrates, 73.0 ± 0.4%, and 76.1 ± 0.2% of d.m.; dietary fibre, 3.3 ± 0.01%, and 1.5 ± 0.03% of d.m.; fat 0.7 ± 0.01%, and 1.0 ± 0.02% of d.m. Three different batches of each flour were pooled and used to prepare the respective sourdoughs. Soft or durum flour (62.5 g) and tap water (37.5 mL), containing the above cellular suspension of each yeast (cell density in the dough of ca. 6.0 Log cfu/g), were used to prepare 100 g of dough (dough yield, DY, dough weight × 100/flour weight, 160) supplemented with chloramphenicol (0.1 g/L). Mixing was done manually for 5 min. Doughs were fermented at 25 °C for 24 h, according to the common temperature used in sourdough preparation at artisanal and industrial levels (Minervini et al., 2012).

Kinetics of growth were determined and modelled in agreement with the Gompertz equation, as modified by Zwietering et al. (1990): $y = k + A \exp\{-\exp[(\mu max \text{ or } Vmax\ e/A)(\lambda - t) + 1]\}$; where y is the growth expressed as Log cfu/g/h at the time t; k is the initial level of the dependent variable to be modelled (Log cfu/g); A is the cell density variation (between inoculation and the stationary phase); μmax is the maximum growth rate expressed as ΔLog cfu/g/h; λ is the length of the lag phase measured in hours. pH values of the dough prior and after the fermentation were determined on-line by a pHmeter (Model 507, Crison, Milan, Italy) with a food penetration probe. The experimental data were modelled by the non-linear regression procedure of the Statistica 12.0 software (Statsoft, Tulsa, USA).

During fermentation, the increase of the volume was manually monitored every two hours and the time of fermentation (t, min) were recorded. The increase of volume (ΔV, cm^3) was calculated as the difference between the volume of the dough at the end and beginning of fermentation. The leavening performance was determined in term of ΔV/t (cm^3/h) (Minervini et al., 2011).

Water/Salt-soluble Extracts (WSE) of doughs and fermented doughs were prepared according to Weiss et al. (1993) and used to analyse Total Free Amino Acids (TFAA) and phytase activity.

TFAA were analysed by a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, England) with a Na-cation-exchange column (20 by 0.46 cm internal diameter), as described by Rizzello et al. (2010).

Not inoculated doughs, prior (CT_0) and after (CT_{24}) the incubation and Lievitalia commercial baker's yeast (CT_C) were used as controls.

2.5. Nutritional and functional properties of selected strains

Phytase activity was determined on the WSE of doughs and fermented doughs, by monitoring the rate of hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP) (Sigma, 104-0). The assay mixture contained 200 μL of 1.5 mM *p*-NPP (final concentration) in 0.2 M Na-acetate, pH 5.2, and 400 μL of WSE. The mixture was incubated at 45 °C and the reaction was stopped by adding 600 μL of 0.1 M NaOH. The *p*-nitrophenol released was determined by measuring the absorbance at 405 nm (Rizzello et al., 2010). One unit (U) of activity was defined as the amount of enzyme required to liberate 1 μmol/min of *p*-nitrophenol under the assay conditions.

Total phenols concentration and antioxidant activity were determined on the Methanolic Extract (ME) of doughs and fermented doughs. Five grams of each dough were mixed with 50 mL of 80% methanol to get ME. The mixture was purged with nitrogen stream for 30 min, under stirring condition, and centrifuged at 4600 × *g* for 20 min. ME were transferred into test tubes, purged with nitrogen stream and stored at ca. 4 °C before analysis. The concentration of total phenols was determined as described by Slinkard and Singleton (1997) and expressed as gallic acid equivalent. The free radical scavenging capacity was determined as reported by Yu et al. (2003) and expressed as follows: DPPH scavenging activity (%) = [(blank absorbance − sample absorbance)/blank absorbance] × 100. The value of absorbance was compared with 75 ppm Butylated HydroxyToluene (BHT), which was used as the antioxidant reference. Not inoculated doughs, prior (CT_0) and after (CT_{24}) the incubation and Lievitalia commercial baker's yeast (CT_C) were used as controls.

2.6. Resistance to simulated gastric and intestinal fluids

Simulated gastric and intestinal fluids were used as described by Fernández et al. (2003). Stationary-phase-grown cells were harvested at 8,000 × *g* for 10 min, washed with physiologic solution, and suspended in 50 mL of simulated gastric juice (ca. 6–7 Log cfu/mL) which contained NaCl (125 mM), KCl (7 mM), $NaHCO_3$ (45 mM), and pepsin (3 g/L) (EC number 3.4.23.1) (Sigma–Aldrich CO., St. Louis, MO, USA) (Zárate et al., 2000). The final pH was adjusted to 2.0, 3.0, and 8.0. The value of pH 8.0 was used to investigate the influence of the components of the simulated gastric juice apart from the effect of low pH (Fernández et al., 2003). The suspension was incubated at 37 °C under anaerobic conditions and agitation to simulate peristalsis. Aliquots of this suspension were taken at 0, 90, and 180 min, and viable count was determined. The effect of gastric digestion was also determined by suspending cells in Reconstituted Skimmed Milk (RSM) (10% solids, *w/v*) before inoculation of simulated gastric juice at pH 2.0. The final pH after the addition of RSM was ca. 3.0. This condition was assayed to simulate the effect of the food matrix during gastric transit (Zárate et al., 2000). After 180 min of gastric digestion, cells were harvested and suspended in simulated intestinal fluid which contained 0.1% (*w/v*) pancreatin and 0.15% (*w/v*) Oxgall bile salt (Sigma–Aldrich Co.) at pH 8.0. The suspension was incubated at 37 °C under agitation and aliquots were taken at 0, 90, and 180 min (Fernández et al., 2003).

2.7. Statistical analysis

Analyses were carried out in triplicate with three biological replicates for each condition. Data were subjected to one-way ANOVA; pair-comparisons of treatment means were achieved by Tukey's procedure at $p < 0.05$, using the statistical software Statistica 7.0 (Statsoft). The normalized parameters values of the growth and acidification kinetics were subjected to the permutation analysis using PermutMatrix (version 1.9.3).

3. Results

3.1. Microbiological analysis and isolation of yeasts from Tuscan sourdoughs

Microbiological analysis showed that yeast counts were not affected by the medium used. The results of yeast viable counts were 6.95 ± 0.10, 5.47 ± 0.02, 6.84 ± 0.14, 7.48 ± 0.02 Log cfu/g in the bakery C, D, G and L, respectively. A total of 200 yeasts were isolated and stored in 20% glycerol at −80 °C. All the isolates were inserted in IMA collection. Among the 200 isolates, 78 were randomly selected and molecularly characterized.

3.2. Molecular identification of isolates

The 78 selected yeast isolates were identified using PCR-RFLP analysis of the ITS region. An amplicon of about 850 bp was obtained for 77 isolates and one of about 650 bp for the isolate IMA G23Y. The amplified products were subjected to restriction analysis using *Hae*III and *Hpa*II enzymes. Amplicons of 850 bp were digested with *Hae*III and *Hpa*II, generating profiles of 310, 230, 165 and 130 bp and 700 and 130 bp, respectively. Such profiles corresponded to those of *S. cerevisiae* (Fernàndez-Espinar et al., 2000). Digestion of IMA G23Y amplicon with *Hpa*II and with *Hae*III generated two fragments of 450 and 180 bp and a profile of 400 and 230 bp, respectively. Such isolate showed the same restriction pattern of *K. humilis* DBVPG 6753^T. The identification of IMA G23Y as *K. humilis* was confirmed by sequencing ITS1-5.8S-ITS2 and 26S D1/D2 rRNA gene amplicons as reported in Palla et al. (2017). The sequences were submitted to the European Nucleotide Archive under the accession numbers of LS974436 and LS420057.

3.3. Molecular characterization at strain level

The preliminary test performed using 5 different molecular techniques identified the inter-delta regions amplification as the best discriminating one (results not shown). Such a method showed a discriminatory index of 0.90, resulting in a powerful differentiation tool for molecular typing of *S. cerevisiae*, and was used for screening the 78 yeast isolates, along with 3 reference strains. The dendrogram, obtained comparing the inter-delta profiles, showed the occurrence of 18 *S. cerevisiae* biotypes (Fig. 1) with a high degree of variability, 23%, measured as the percentage of different biotypes found *vs.* the number of isolates analysed. None of our isolates shared the profiles with the two commercial baker's yeast strains analysed.

One strain representative of each biotype group of the yeasts isolated from the 4 different sourdoughs (16 biotypes) was selected and analysed, along with *K. humilis* G23Y strain and 3 *S. cerevisiae* belonging to the IMA collection (Fig. 1; Table 1).

3.4. Pro-technological, nutritional and functional characterization of yeast strains

The 20 selected strains were singly used to ferment soft and durum wheat flour dough at 30 °C for 24 h. Overall, all strains were able to grow. The cell growth during incubation corresponded to ca. 2 Log cycles for all strains considered. Strains showed values of ΔLog cfu/g in the range of 2.0 ± 0.2 (G23Y)–2.7 ± 0.1 (D5Y) and 2.0 ± 0.3 (G23Y)–2.9 ± 0.2 (D9Y) in soft and durum fermented dough, respectively (Table 2). The median values of the λ was 2.0 ± 0.2 h in both soft and durum fermented dough. Means of pH values at the end of the fermentation were 5.64 ± 0.31 and 5.59 ± 0.22 in soft and durum fermented dough, respectively. CT_{24} showed a slight increase of yeast cell density (2.4 Log cfu/g).

The increase of volume was monitored during the fermentation. Although lower than the CT_C, doughs reached the highest volumes after 16 h of fermentation in both soft and durum fermented dough when the yeast strains were used. The ΔV varied from 9.4 to 12.6 cm^3 and from 6.2 to 9.4 cm^3, when soft and durum flours were used, respectively. Fermented doughs started with *K. humilis* G23Y showed the worst leavening performances (0.4 and 0.5 cm^3/h) in both soft and durum fermented dough. However, *S. cerevisiae* D2Y showed the best rate in soft fermented dough (0.8 cm^3/h). The use of soft flours guaranteed higher increase of volume during the fermentation compared to the corresponding durum dough. As expected, CT_{24} showed the lowest increase of volume after 16 h of fermentation (3.2 cm^3) regardless the type of wheat flour used.

The use of *S. cerevisiae* strains to ferment soft and durum wheat flours led to a decrease of the TFAA. Overall, decreases of TFAA were in the range of 48 ± 4% (D18Y)–84 ± 6% (D2Y) and 30 ± 2% (D9Y)–79 ± 5% (36Y), in soft and durum fermented dough, respectively (Table 2). The dough CT_{24} was characterized by the lowest decrease of the TFAA, however the use of commercial baker's yeast (CT_C) led to a significant decrease of the concentrations (Table 2). Among others, valine (Val), asparagine (Asp), γ-Aminobutyric acid (GABA) and tryptophan (Trp) were the free amino acids mostly affected by the fermentation. Decreases were > 80% in almost all fermented doughs started with *S. cerevisiae* strains as compared to the CT_0 (Supplementary Table S1). With few exceptions (strains D2Y, D4Y, D5Y, L25Y and 19Y), where glutamic acid (Glu) and cysteine (Cys) decreased in soft wheat fermented doughs, all the other *S. cerevisiae* strains determined an increase of these two free amino acids Glu and Cys reaching values up to 7 folds higher than the CT_0 (Supplementary Table S1). A different behavior was found for *K. humilis* G23Y, which caused an increase of TFAA of 59 ± 3% and 58 ± 5% in soft and durum fermented dough, respectively (Table 2). Alanine (Ala), Val, isoleucine (Ile) and tyrosine (Tyr) were the most increased FAA in soft wheat fermented doughs as well as threonine (Thr), Ala, Ile and leucine (Leu) were the most affected in durum fermented doughs (Supplementary Table S1).

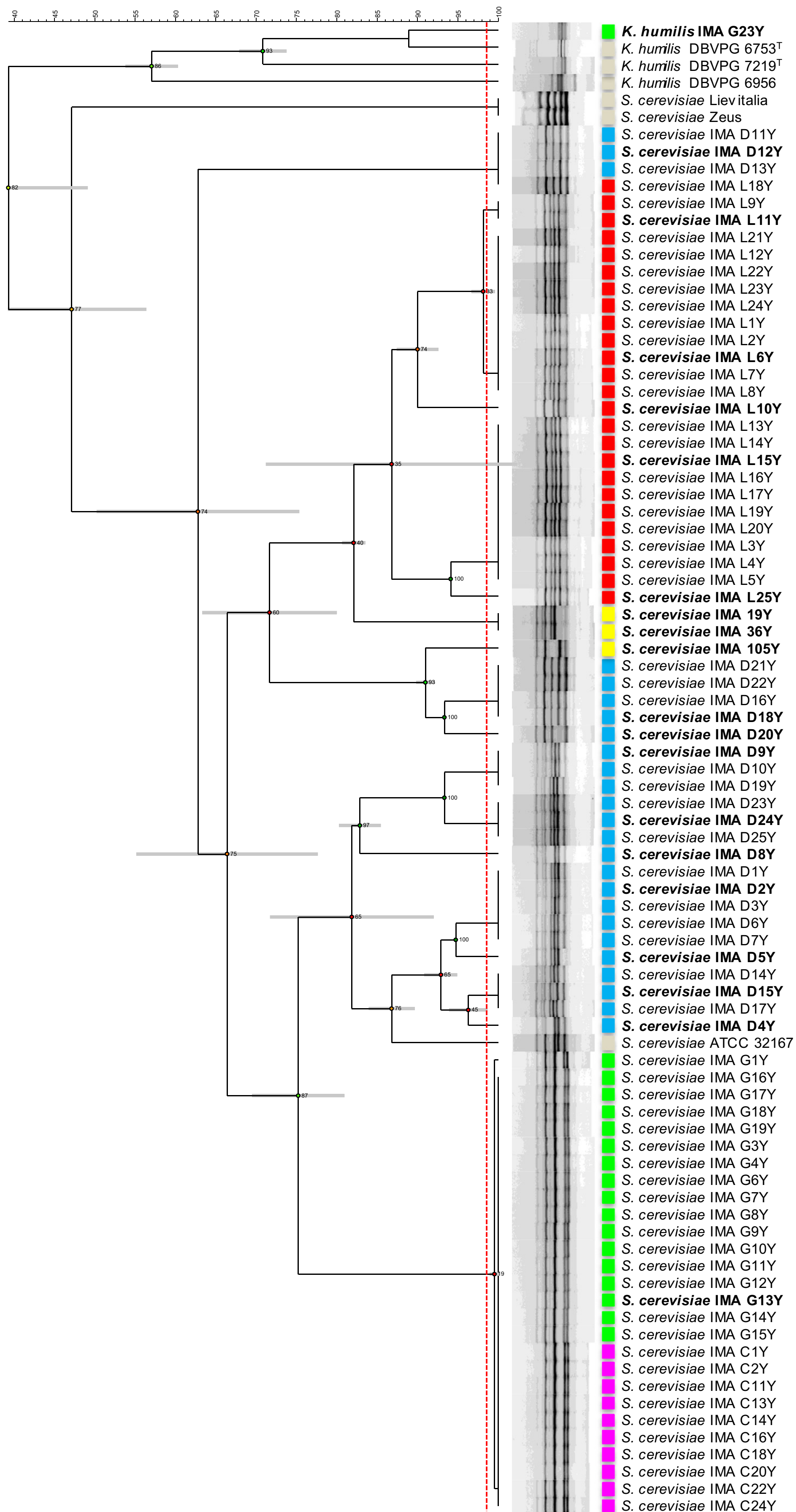
After fermentation, also phytase activity was measured. Overall, values of phytase activity were significantly ($p < 0.05$) higher in durum compared to the corresponding soft fermented dough. Moreover, *S. cerevisiae* D20Y, D24Y and L6Y and *K. humilis* G23Y exhibited phytase activity up to 65% and 74% higher than CT_{24} in soft and durum fermented dough, respectively (Table 2). Although lower than the mean value found in the started doughs, the phytase activity of the CT_C was slightly, but significantly higher than the CT_0 and CT_{24}.

S. cerevisiae D20Y and D24Y and *K. humilis* G23Y showed the highest concentration of total phenols in both soft and durum flours (Table 2). Although it was significantly ($p < 0.05$) lower than the reference (BHT, ca. 65%), strains of *S. cerevisiae* and *K. humilis* showed high (> 30%) antioxidant activity, regardless the type of flour used to make fermented dough (Table 2). The use of commercial baker's yeast (CT_C) did not significantly affect the concentration of total phenols, however an increase of the antioxidant activity was found (Table 2).

All strains of *S. cerevisiae* and *K. humilis* showed a marked high resistance when incubated under simulated gastric and intestinal conditions (Fig. 2A–D). The decrease of cell density in the most hostile condition (pH 2.0) was up to 1.5 Log cfu/mL (19Y). *S. cerevisiae* D15Y, D9Y and D4Y did not show any decrease of cell density in any conditions at any time.

4. Discussion

After the identification and classification of numerous species of sourdough yeasts from cereal fermentations (Huys et al., 2013), basic and applied sciences are currently facing the challenge to identify functional traits of these microbes to fully exploit their potential. Here, we investigated the pro-technological, nutritional and functional traits of sourdough yeast microbiota isolated from Tuscan sourdoughs and selected the best performing strains, to be used as starters for making sourdough baked goods or functional cereal-based fermented beverages (Fig. 3).



(caption on next page)

Fig. 1. Dendrogram obtained from UPGMA analysis, using Dice's coefficient, based on inter-delta profiles of 78 sourdough yeast isolates, 7 reference strains and 2 commercial baker's yeasts. The red line indicates the similarity value (98.7%) for separation of biotypes. Cophenetic correlation is shown at each branch by numbers and coloured dots, ranging between green-yellow-orange-red, according to decreasing values. Standard deviation is shown at each node by a grey bar. The 20 selected strains are reported in bold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Among the 200 yeasts isolated from the four Tuscan sourdoughs, 78 isolates were randomly selected and molecularly characterized by using ITS-RFLP analysis. *S. cerevisiae* was the dominating yeast, representing the only species detected in three out of four sourdoughs. These results are consistent with previous studies reporting *S. cerevisiae* as the most frequently isolated species in sourdoughs from central and southern Italy (Iacumin et al., 2009; Minervini et al., 2012; Osimani et al., 2009; Ricciardi et al., 2005; Valmorri et al., 2010).

In this study, *K. humilis* occurred only in one bakery, in agreement with previous studies carried out on the PDO Tuscan bread sourdough, where *K. humilis* and *S. cerevisiae* were found in association with *L. sanfranciscensis* (Palla et al., 2017). Italian bakery sourdoughs often harbor *K. humilis* and/or *S. cerevisiae* as dominating yeasts (De Vuyst et al., 2016). The inter-delta regions analysis, a method providing high strain differentiation levels, allowed the discrimination of 16 biotypes among the 77 *S. cerevisiae* isolates, revealing a high intraspecific genetic

Table 2
Pro-technological properties (Δvolume rate and kinetics of growth), Total Free Amino Acids (TFAA) and phenols concentrations, phytase and radical scavenging activities of soft and durum wheat flour doughs individually inoculated (cell density of ca. 6 Log cfu/g) with selected yeast strains and fermented at 25 °C for 24 h. Data are the means of three independent analyses. Not inoculated doughs prior (CT_0) and after (CT_{24}) the fermentation and commercial baker's yeast (CT_C), are reported.

	Pro-technological properties				Functional features			
	ΔV rate (cm^3/h)	A (ΔLog cfu/g)	μmax (ΔLog cfu/h)	Λ (h)	TFAA (mg/kg)	Phytase activity (U)	Total phenols (mgAGE/g)	Radical scavenging activity (%)
Soft wheat flour dough								
CT_0	n.d.	n.d.	n.d.	n.d.	942 ± 10^{b}	4.87 ± 0.04^{m}	0.08 ± 0.01^{m}	7.62 ± 0.09^{q}
CT_{24}	0.18 ± 0.01^{f}	1.34 ± 0.07^{o}	0.52 ± 0.04$^{g–i}$	3.7 ± 0.21^{a}	821 ± 8^{c}	5.54 ± 0.05^{m}	0.09 ± 0.02^{l,m}	8.54 ± 0.12^{p}
CT_C	1.73 ± 0.11^{a}	2.71 ± 0.05^{a,b}	1.11 ± 0.05^{a}	0.48 ± 0.05^{d}	242 ± 1^{n}	6.52 ± 0.09^{l}	0.14 ± 0.05^{l,m}	24.12 ± 0.35^{h}
L6Y	0.81 ± 0.05^{b,c}	2.19 ± 0.08^{m}	0.93 ± 0.04$^{b–d}$	2.28 ± 0.14^{b}	319 ± 5^{l}	15.41 ± 0.05^{a}	0.37 ± 0.02$^{f–h}$	32.51 ± 0.24^{a}
L10Y	0.79 ± 0.08$^{b–d}$	2.57 ± 0.05$^{b–h}$	0.31 ± 0.01^{l}	0.53 ± 0.03^{d}	382 ± 7^{h}	13.82 ± 0.04^{b}	0.32 ± 0.01^{g,h}	29.22 ± 0.26^{c}
L11Y	0.73 ± 0.09$^{b–e}$	2.51 ± 0.07$^{c–h}$	0.39 ± 0.01 ^{i,l}	1.34 ± 0.06^{c}	331 ± 6^{l}	6.12 ± 0.07^{l}	0.14 ± 0.01^{l,m}	27.32 ± 0.31^{f}
L15Y	0.72 ± 0.04$^{b–e}$	2.39 ± 0.07$^{g–l}$	0.60 ± 0.03$^{f–h}$	2.06 ± 0.21^{b}	464 ± 4^{f}	5.92 ± 0.03^{l}	0.16 ± 0.02^{l,m}	23.02 ± 0.22^{i,l}
L25Y	0.69 ± 0.06$^{b–e}$	2.37 ± 0.06$^{h–m}$	0.44 ± 0.05$^{h–l}$	1.97 ± 0.12^{b}	262 ± 9^{n}	7.47 ± 0.07^{h}	0.13 ± 0.01^{l,m}	28.61 ± 0.34^{c,d}
D2Y	0.82 ± 0.03^{b}	2.69 ± 0.04$^{a–c}$	0.96 ± 0.02$^{a–c}$	2.36 ± 0.16^{b}	146 ± 7^{p}	8.95 ± 0.04$^{e–f}$	0.19 ± 0.02^{i,l}	26.30 ± 0.32^{g}
D4Y	0.71 ± 0.06$^{b–e}$	2.58 ± 0.05$^{a–f}$	0.61 ± 0.06$^{f–g}$	2.00 ± 0.12^{b}	546 ± 6^{d}	9.44 ± 0.08^{e}	0.29 ± 0.02^{h,i}	18.57 ± 0.27^{m}
D5Y	0.75 ± 0.07$^{b–e}$	2.73 ± 0.06^{a,b}	0.45 ± 0.04$^{g–l}$	1.37 ± 0.10^{c}	199 ± 3^{o}	8.65 ± 0.02$^{f–g}$	0.31 ± 0.04^{h}	22.48 ± 0.22^{l}
D8Y	0.74 ± 0.07$^{b–e}$	2.67 ± 0.09$^{a–d}$	0.46 ± 0.07$^{g–l}$	1.37 ± 0.14^{c}	321 ± 4^{l}	9.44 ± 0.05^{e}	0.29 ± 0.01^{h,i}	27.36 ± 0.28^{f}
D9Y	0.74 ± 0.02$^{b–e}$	2.64 ± 0.08$^{a–e}$	0.71 ± 0.08$^{e–f}$	1.99 ± 0.21^{b}	375 ± 6^{h,i}	9.04 ± 0.09$^{e–f}$	0.42 ± 0.04$^{e–g}$	17.27 ± 0.19^{n}
D12Y	0.71 ± 0.06$^{b–e}$	2.45 ± 0.05$^{f–i}$	0.42 ± 0.03 ^{i,l}	1.49 ± 0.12^{c}	290 ± 4^{m}	7.47 ± 0.02^{h}	0.17 ± 0.02^{l,m}	27.18 ± 0.21^{f}
D15Y	0.65 ± 0.07$^{c–e}$	2.55 ± 0.06$^{b–h}$	0.92 ± 0.08$^{b–d}$	2.19 ± 0.23^{b}	245 ± 2^{n}	8.32 ± 0.08^{g}	0.14 ± 0.01^{l,m}	27.66 ± 0.29^{e,f}
D18Y	0.64 ± 0.04^{d,e}	2.47 ± 0.03$^{e–h}$	0.31 ± 0.02^{l}	1.27 ± 0.14^{c}	492 ± 5^{e}	8.76 ± 0.05$^{f–g}$	0.66 ± 0.05^{a}	23.33 ± 0.25^{h,i}
D20Y	0.79 ± 0.02$^{b–d}$	2.50 ± 0.07$^{d–h}$	0.31 ± 0.06^{l}	1.35 ± 0.17^{c}	245 ± 7^{n}	15.35 ± 0.07^{a}	0.54 ± 0.06$^{b–d}$	22.82 ± 0.21^{i,l}
D24Y	0.78 ± 0.00$^{b–d}$	2.38 ± 0.03$^{h–l}$	1.12 ± 0.03^{a}	2.36 ± 0.13^{b}	355 ± 2^{i}	15.72 ± 0.08^{a}	0.49 ± 0.04$^{c–e}$	28.22 ± 0.31^{d,e}
G13Y	0.61 ± 0.04^{e}	2.74 ± 0.09^{a}	0.48 ± 0.08$^{g–i}$	1.46 ± 0.14^{c}	428 ± 5^{g}	6.73 ± 0.08^{i}	0.45 ± 0.05$^{d–f}$	31.31 ± 0.32^{b}
G23Y	0.59 ± 0.05^{e}	1.96 ± 0.04^{n}	0.83 ± 0.07$^{c–e}$	2.33 ± 0.11^{b}	1499 ± 11^{a}	15.52 ± 0.17^{a}	0.52 ± 0.06$^{b–e}$	17.93 ± 0.18^{m,n}
19Y	0.75 ± 0.04$^{b–e}$	2.23 ± 0.09^{l,m}	0.78 ± 0.05$^{d–e}$	2.19 ± 0.09^{b}	154 ± 3^{p}	12.26 ± 0.15^{d}	0.58 ± 0.04$^{a–c}$	28.52 ± 0.27^{c,d}
36Y	0.71 ± 0.06$^{b–e}$	2.28 ± 0.01$^{i–m}$	0.60 ± 0.03$^{f–h}$	1.97 ± 0.11^{b}	377 ± 4^{h}	12.79 ± 0.08^{c}	0.14 ± 0.01^{l,m}	22.74 ± 0.24^{i,l}
105Y	0.72 ± 0.07$^{b–e}$	2.51 ± 0.01$^{c–h}$	1.01 ± 0.11^{a,b}	2.34 ± 0.13^{b}	389 ± 8^{h}	13.53 ± 0.12^{b}	0.61 ± 0.05^{a,b}	11.85 ± 0.14^{o}
Durum wheat flour dough								
CT0	n.d.	n.d.	n.d.	n.d.	1010 ± 16^{b}	7.69 ± 0.08^{p}	0.15 ± 0.01^{l}	9.62 ± 0.05^{q}
CT24	0.12 ± 0.03^{l}	1.22 ± 0.11^{l}	0.54 ± 0.06$^{f–h}$	4.03 ± 0.79^{a}	947 ± 10^{c}	8.81 ± 0.09^{o}	0.15 ± 0.02^{l}	10.50 ± 1.11^{q}
CT_C	1.54 ± 0.05^{a}	2.84 ± 0.14^{a,b}	1.05 ± 0.10^{a}	0.66 ± 0.06^{h,i}	275 ± 13	10.22 ± 1.09^{n}	0.18 ± 0.06^{i,l}	38.21 ± 0.67^{h}
L6Y	0.61 ± 0.04^{b,c}	2.36 ± 0.08^{g,h}	0.80 ± 0.03$^{c–e}$	2.15 ± 0.11$^{b–d}$	577 ± 7^{g,f}	21.29 ± 0.22^{c}	0.23 ± 0.05 $^{g–l}$	33.61 ± 0.41^{e}
L10Y	0.54 ± 0.03$^{e–g}$	2.58 ± 0.05$^{c–f}$	0.36 ± 0.01^{i,l}	1.38 ± 0.15$^{e–h}$	577 ± 6^{g,f}	14.29 ± 0.16^{h,i}	0.51 ± 0.07^{c}	32.23 ± 0.39^{f}
L11Y	0.52 ± 0.06$^{f–h}$	2.38 ± 0.06$^{f–h}$	0.87 ± 0.04$^{b–d}$	2.26 ± 0.25^{b,c}	565 ± 4^{h,g}	14.22 ± 0.11^{i,h}	0.19 ± 0.02$^{h–l}$	39.55 ± 0.37^{b}
L15Y	0.55 ± 0.06^{e,f}	2.55 ± 0.06$^{c–g}$	0.63 ± 0.05$^{e–g}$	1.95 ± 0.12 $^{b–e}$	508 ± 9^{l,m}	15.03 ± 0.13^{g,h}	0.40 ± 0.01^{d,e}	36.48 ± 0.35^{d}
L25Y	0.56 ± 0.07^{d,e}	2.52 ± 0.09$^{d–g}$	0.53 ± 0.07$^{f–i}$	1.93 ± 0.18 $^{b–e}$	574 ± 8$^{h–f}$	18.36 ± 0.17^{d}	0.55 ± 0.06^{b,c}	41.69 ± 0.29°
D2Y	0.63 ± 0.02^{b}	2.63 ± 0.03$^{b–e}$	0.77 ± 0.07^{d,e}	2.34 ± 0.21^{b,c}	490 ± 4^{m,n}	12.86 ± 0.11^{l}	0.24 ± 0.01$^{g–l}$	21.68 ± 0.24^{m}
D4Y	0.49 ± 0.04^{h}	2.64 ± 0.00$^{b–e}$	0.72 ± 0.03^{d,e}	2.18 ± 0.23 $^{b–d}$	349 ± 5^{o}	13.66 ± 0.15^{i,l}	0.40 ± 0.02$^{g–l}$	20.37 ± 0.27^{n}
D5Y	0.51 ± 0.05^{g,h}	2.73 ± 0.03$^{a–d}$	0.48 ± 0.04$^{f–l}$	1.47 ± 0.14$^{d–g}$	596 ± 6^{f}	11.48 ± 0.12^{m}	0.30 ± 0.04 $^{e–g}$	23.0 3 ± 0.32^{i,l}
D8Y	0.52 ± 0.03$^{f–h}$	2.75 ± 0.02$^{a–c}$	0.47 ± 0.01$^{g–l}$	1.62 ± 0.17$^{c–f}$	250 ± 5^{q}	12.89 ± 0.12^{l}	0.20 ± 0.01$^{g–l}$	18.80 ± 0.11^{o}
D9Y	0.59 ± 0.04^{c,d}	2.87 ± 0.05^{a}	0.48 ± 0.06$^{f–l}$	1.23 ± 0.11$^{e–h}$	702 ± 8^{d}	12.98 ± 0.14^{l}	0.23 ± 0.02 $^{g–l}$	30.62 ± 0.35^{g}
D12Y	0.54 ± 0.03$^{e–g}$	2.51 ± 0.05$^{e–h}$	0.88 ± 0.08$^{a–d}$	2.20 ± 0.22^{b,c}	224 ± 4^{r}	12.81 ± 0.14^{l}	0.30 ± 0.01$^{e–g}$	30.71 ± 0.31^{g}
D15Y	0.55 ± 0.06^{e,f}	2.61 ± 0.04$^{c–e}$	0.83 ± 0.09^{c,d}	2.15 ± 0.23 $^{b–d}$	637 ± 8^{e}	15.56 ± 0.16^{f,g}	0.28 ± 0.01$^{f–i}$	22.04 ± 0.26^{l,m}
D18Y	0.54 ± 0.02$^{e–g}$	2.62 ± 0.09$^{c–e}$	0.34 ± 0.02^{l}	0.76 ± 0.08^{g,h}	532 ± 6^{i,l}	23.82 ± 0.21^{b}	0.38 ± 0.02^{e,f}	12.05 ± 0.13^{p}
D20Y	0.59 ± 0.02^{c,d}	2.56 ± 0.08$^{c–g}$	0.34 ± 0.01^{l}	0.91 ± 0.09$^{f–h}$	552 ± 6^{i,h}	33.57 ± 0.27^{a}	0.63 ± 0.01^{a}	33.44 ± 0.31^{e,f}
D24Y	0.59 ± 0.02^{c,d}	2.67 ± 0.06$^{a–e}$	0.37 ± 0.01$^{h–l}$	1.18 ± 0.11$^{f–h}$	579 ± 7^{g,f}	23.82 ± 0.19^{b}	0.71 ± 0.05^{a}	29.51 ± 0.36^{g}
G13Y	0.51 ± 0.05^{g,h}	2.54 ± 0.04$^{c–g}$	0.97 ± 0.05$^{a–c}$	2.29 ± 0.22^{b,c}	480 ± 4^{n}	11.65 ± 0.12^{m}	0.49 ± 0.04^{c,d}	38.72 ± 0.41^{b,c}
G23Y	0.44 ± 0.06^{i}	2.03 ± 0.07^{i}	1.04 ± 0.11$^{a–b}$	2.43 ± 0.23^{b}	1598 ± 12^{a}	18.93 ± 0.22^{d}	0.73 ± 0.05^{a}	33.22 ± 0.25^{e,f}
19Y	0.56 ± 0.04^{d,e}	2.30 ± 0.09^{h}	0.65 ± 0.05^{e,f}	2.12 ± 0.24 $^{b–d}$	567 ± 6^{h,g}	16.27 ± 0.21^{e,f}	0.29 ± 0.02$^{f–h}$	33.43 ± 0.21^{e,f}
36Y	0.55 ± 0.05^{e,f}	2.55 ± 0.02$^{c–g}$	0.43 ± 0.02$^{h–l}$	1.34 ± 0.15$^{e–h}$	213 ± 5^{r}	16.09 ± 0.19^{e,f}	0.16 ± 0.02^{l}	37.54 ± 0.44^{c,d}
105Y	0.54 ± 0.03$^{e–g}$	2.64 ± 0.09$^{b–e}$	0.96 ± 0.06$^{a–c}$	2.30 ± 0.05^{b,c}	643 ± 8^{e}	16.68 ± 0.15^{e}	0.57 ± 0.03^{c,d}	23.34 ± 0.22^{i}

n.d., not detectable; $^{a–r}$Values in the same column with different superscript letters differ significantly ($p < 0.05$).

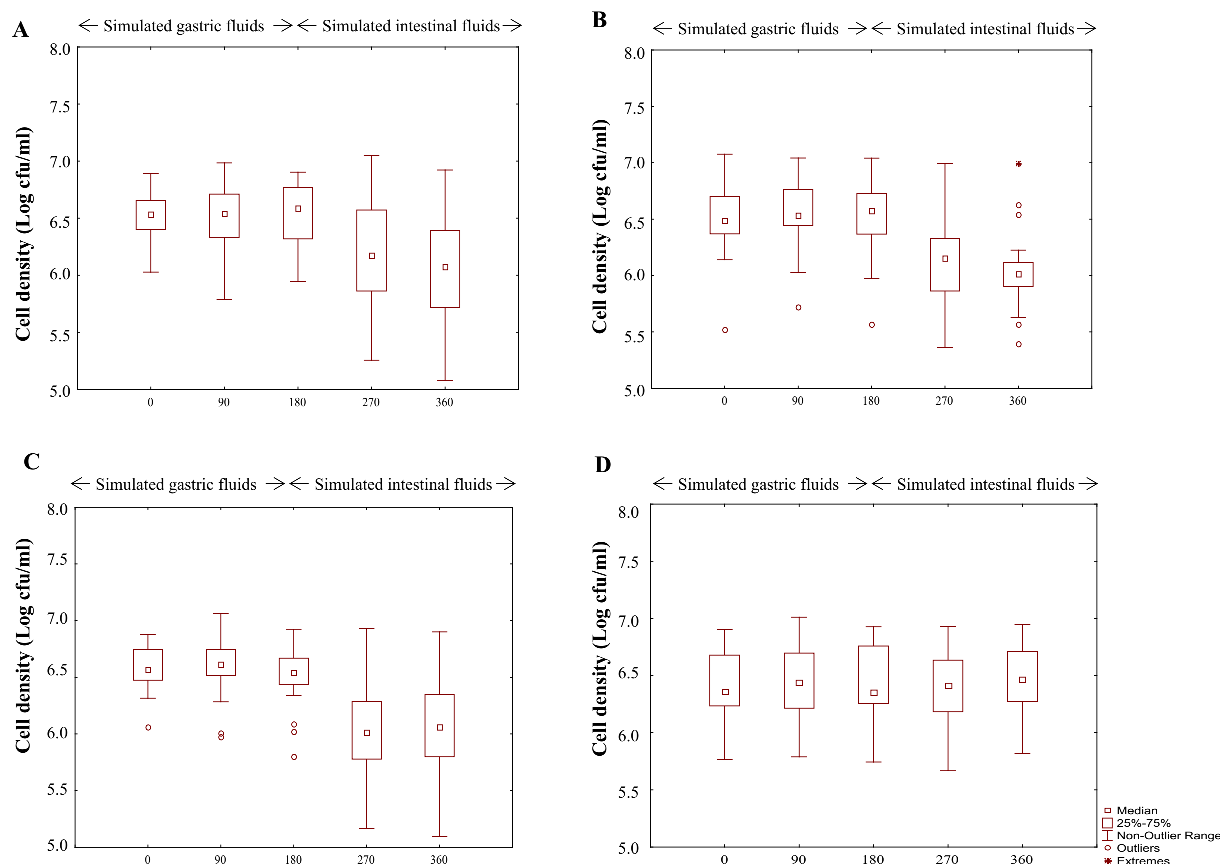


Fig. 2. Boxplot showing the survival of 19 strains of *Saccharomyces cerevisiae* and 1 strain *Kazachstania humilis* under gastric conditions (0–180 min) at pH 2.0 (A), 3.0 (B), 8.0 (C), and 2.0 with reconstituted skim milk added (11%) (D), and further intestinal digestion (180–360 min) at pH 8.0. Median values are represented (■). The top and the bottom of the box represent the 75th and 25th percentile of the data, respectively. The top and the bottom of the bars represent the 5th and the 95th percentile of the data, respectively.

polymorphism (De Vuyst et al., 2016; Landry et al., 2006; Osimani et al., 2009; Pulvirenti et al., 2001). None of our yeast isolates belonged to the same biotypes of *S. cerevisiae* collection strains and commercial baker's yeasts. Moreover, cluster analysis of the inter-delta regions profiles showed, that *S. cerevisiae* isolates originating from the different bakeries were genetically diverse, grouping according to their origin. A wide biotype diversity was found in bakeries D and L, where 10 and 6 biotypes, out of 25 isolates each, were detected, respectively. Such diversity suggests that our *S. cerevisiae* isolates may be native, deriving from raw material and bakery environment, and not from possible working environment cross contamination by baker's yeast.

A general protocol for yeast selection was proposed (Pulvirenti et al., 2009) as follows: i) selection of yeast strains dominating spontaneous fermentations; ii) selection of the most pro-technologically relevant strains; and iii) selection and validation of the most promising strains. Here, we selected 20 yeast isolates: one representative of each biotype, along with *K. humilis* G23Y strain and three *S. cerevisiae* belonging to the IMA collection, in order to further assess their pro-technological, nutritional and functional properties when inoculated in soft and durum wheat flour.

During fermentation under standardized conditions, all strains were able to grow of ca. 2 Log cycles, however only three strains of *S. cerevisiae* isolated from two bakeries (L and D) had a significantly shorter latency phase in both the flours. Overall, all yeast strains reached a final cell density of ca. 8 Log cfu/g after 16 h of fermentation. These results are in accordance with values usually found in wheat type-I sourdough (Minervini et al., 2012). Volume is one of the organoleptic quality parameters of breads (Katina et al., 2006), therefore strains were characterized for their leavening capacity. Six out of 20 strains caused high dough volume increases and all belonged to *S. cerevisiae* isolated from bakery D sourdough. Best leavening performances were found when a commercial baker's yeast was used to ferment both soft and durum doughs. These results confirmed the high suitability of such yeast for the preparation of baked goods. As widely known, proteolysis during sourdough fermentation provides amino acids for microbial growth and for the production of flavour precursor compounds and antifungal metabolites (Gobbetti et al., 2005; Hansen and Schieberle, 2005). Consequently, the effect of yeasts on the level of TFAA was investigated. All *S. cerevisiae* strains determined a decrease of the TFAA in fermented dough made with both flour types. The magnitudes of changes in amino acids composition and concentration was strain-dependent, however, some amino acids were similarly and abundantly (> 80%) metabolized (Val, Asp, GABA and Trp) or released (Glu and Cys) during fermentation in almost all samples. As widely reported, catabolism of free amino acids during sourdough fermentation carried out by added *Saccharomyces* and *Hansenula* yeasts resulted in increased synthesis of volatile compounds belonging to different chemical classes, such as alcohols, esters and some carbonyl compounds as compared to sourdoughs without added yeast (Damiani et al., 1996; Hansen and Hansen, 1994). These compounds can be formed via the Ehrlich pathway by transamination of branched amino acids (Leu, Ile and Val). *K. humilis* G23Y was the only strain able to increase the TFAA concentration of doughs. Sourdough fermentation is known to contribute in many ways to the enhanced nutritional and functional features of the resulting baked goods (Gobbetti et al., 2014; Rizzello et al., 2016). Microbial activities of both yeasts and LAB contribute differently to the production of health-improving metabolites of cereal substrates. Examples of nutritional improvement based on yeast metabolism

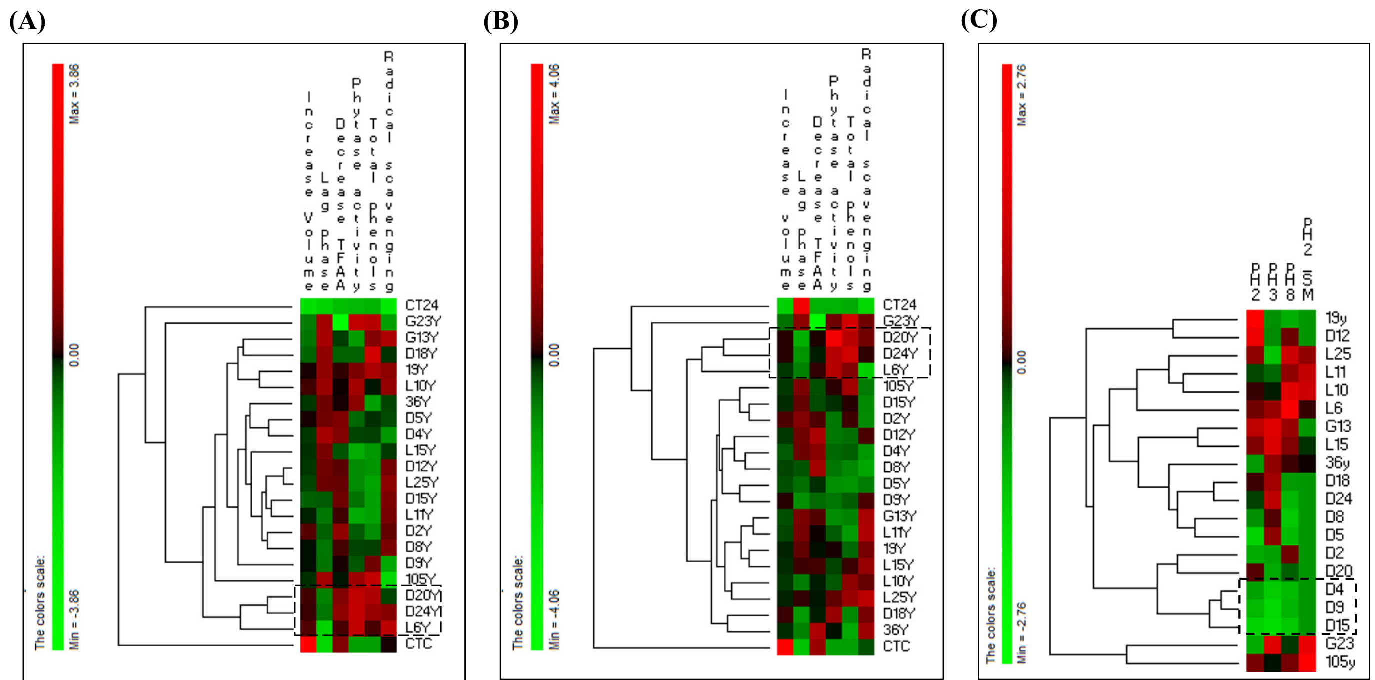


Fig. 3. Increase of volume (cm^3), length of the lag phase (hours) of kinetics of growth, decrease of total free amino acids (mg/kg), phytase activity (U), total phenols (mgAGE/g), radical scavenging (%) of soft (A) and durum (B) wheat fermented dough (DY 160) started with 19 *Saccharomyces cerevisiae* and 1 *Kazachstania humilis* strains. Survival of the yeast strains under gastric conditions (0–180 min) at pH 2.0, 3.0, 8.0, and 2.0 with reconstituted skim milk added (11%) and further intestinal digestion (180–360 min) at pH 8.0 (C). Euclidean distance and McQuitty's criterion (weighted pair group method with averages) were used for clustering. The colours correspond to normalized mean data levels from low (green) to high (red). The colour scale, in terms of units of standard deviation, is shown on the left side. Strains marked by the dashed box are the best performing starters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

encompasses phytase (Türk et al., 2000) and antioxidant (Moore et al., 2007; Wang et al., 2014) activities. Besides, yeasts may exert probiotic properties (Moslehi-Jenabian et al., 2010). Although lower than the flour endogenous enzymes (Reale et al., 2004), all the studied yeast strains showed phytase activity. Overall, it was higher when the durum wheat flour was used. The increase of phytase activity during fermentation, under our experimental conditions, may be ascribed only to yeasts, because the pH values of the dough at the end of the fermentation were far to be optimal for the activation of the endogenous enzymes (Türk et al., 1996). Phytase activity was found in commercial baker's yeasts (Türk et al., 2000), however the values found in CT_C used in this study were only slightly higher than CT_0 and CT_{24}. One strain of *S. cerevisiae* was also found to be able to hydrolyse phytate during bread-making or under simulated Gastro-Intestinal (GI) conditions (Caputo et al., 2015). Here, we found *K. humilis* G23Y and *S. cerevisiae* D20Y, D24Y and L6Y as strains characterized by threefold higher phytase activity than spontaneously fermented control. Yeasts may contribute to the antioxidant capacity of cereal products through their effects on the bio-accessibility and bioavailability of phenolic compounds (Wang et al., 2014). Almost all strains caused an increase of the total phenols concentration in the methanolic extracts, and the fermentation with 13 out of 20 strains led to a significant increase (more than twofold). These data are consistent with those reported in other studies (Đorđević et al., 2010; Liukkonen et al., 2003). Fermentation with *S. cerevisiae* or *K. humilis* had significant influence on DPPH inhibitory effect in soft and durum wheat flours. However, no relevant differences among the strains were found, and no correlation existed between total phenols content and DPPH radical scavenging activity. Doughs with higher total phenols content were not necessarily better in DPPH inhibition. As previously shown, increased contents of total phenolics by yeast activity during cereal fermentation did not always lead to significant anti-oxidative effects (Đorđević et al., 2010). The weak antiradical effect of ferulic acid, the main phenolic acid in cereal grains, might also explain such discrepancies (Brand-Williams et al., 1995).

As a last example of functional improvement, it can be highlighted that certain yeasts may possess probiotic properties, based on potential immunomodulatory effects (Moslehi-Jenabian et al., 2010). *S. cerevisiae* possesses the qualified presumption of safety status (Moslehi-Jenabian et al., 2010) and many authors reported its beneficial effects on human health (Czerucka et al., 2007; Weichselbaum, 2009). In particular, strains of *S. cerevisiae* var. *boulardii* are already commercially used as probiotics (Palma et al., 2015). Here, the resistance of our yeast strains to simulated gastric and intestinal conditions, a pre-requisite for the selection of probiotics, was investigated. High in vitro survival for many of the strains tested was found.

The use of yeasts to ferment plant matrices in substitution to milk was recently purposed in order to obtain non-dairy beverages as an alternative to the traditional dairy products with improved healthy and sensory features (Freire et al., 2017). Cereal-based fermented beverages are an example of non-dairy products, which are considered potential carriers for probiotic strains and alternatives for use by vegans and lactose-intolerant consumers. Functional traits of our yeast strains make them potential starters for novel cereal-based fermented beverages. A mixed starter composed by *S. cerevisiae*, *Pichia kluyveri* and *Lactobacillus paracasei* strains was recently successfully selected for making a functional maize-based beverage (Menezes et al., 2018). Although the experimental conditions tested in this study are far from those that normally characterize the beverages, some highlighted metabolic and, especially pro-technological traits (e.g., growth capability) suggest their potential use as functional starters. An appropriate characterization and selection of microbial strains is necessary to efficiently control the cereal-based beverage fermentation.

Findings encourage the exploitation of our yeast strains for their future pro-technological characterization under typical beverages conditions for the manufacture of innovative cereal-based beverages (De

Vuyst et al., 2016).

5. Future perspectives

Worldwide, the selection of commercial yeast strains suitable for baked goods has mainly been made according to their leavening and technological traits, while disregarding any functional features able to enhance the nutritional and nutraceutical properties of the derived end-products. In this study autochthonous yeast strains isolated from Tuscan sourdoughs showed differential levels of pro-technological and functional activities, compared with a commercial baker's yeasts, representing valuable potential functional starters for biotechnological applications. The properties of the best performing isolates could be exploited to produce sourdough baked goods (Fig. 3A, B) or functional cereal-based beverages (Fig. 3C) and/or to design multifunctional starters, possibly enriched with selected lactic acid bacteria, for the industrial production of innovative, health promoting baked goods. Metabolic traits such the synthesis of volatile compounds, the level of alcohol produced, the co-culture with lactic acid bacteria, and especially the effect on sensory properties are some properties that need to be evaluated to ensure successful fermentations.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.08.004.

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