JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

IDENTIFICATION OF YEAST ISOLATED FROM LABORATORY SOURDOUGHS PREPARED WITH GRAPE, APPLE, AND YOGURT

Elena Gordún[*1], *Anna Puig*[2], *Lola Piñol*[2], *Rosa Carbó*[1]

Address(es): Elena Gordún,
[1] Universitat Politècnica de Catalunya Barcelona Tech, Agricultural Engineering School of Barcelona, Department of Agri-Food Engineering and Biotechnology, c/ Esteve Terradas 8, 08860 Castelldefels, Spain, +34 935521083.
[2] Institute of Agri Food Research and Technology-Catalan Institute of Vine and Wine (IRTA-INCAVI), Department of Oenological Research, Plaça Àgora 1, 08720 Vilafranca del Penedès, Spain.

*Corresponding author: elena.gordun@upc.edu

doi: 10.15414/jmbfs.2018.7.4.399-403

ARTICLE INFO

Received 15. 2. 2017
Revised 15. 12. 2017
Accepted 18. 12. 2017
Published 1. 2. 2018

Regular article

OPEN ACCESS

ABSTRACT

Yeast strains collected from three spontaneous laboratory sourdoughs, prepared over 10 days and using a traditional Spanish protocol, and formulated with (non-essential) different ingredients were identified and described. In addition to wheat flour (*Triticum aestivum*) and water, organic apples (5 days juice fermented at 40°C), or plain yogurt, or organic white grapes (2 days must fermented at 20°C) were used at the beginning of each sourdough production.
One-hundred-eighteen yeast colonies were collected from the different phases of sourdoughs (ingredients preparation, pre-sourdough, and ripe sourdough propagation) using WL agar and Lysine agar media. Yeast isolates were clustered into 8 groups using PCR-RFLP analysis of the 5.8S-ITS rRNA region. One strain of each group was chosen for sequencing to confirm yeast identification at species level. The specifically prepared fruit ingredients, apple and grape, provided diversity of non-*Saccharomyces* yeast to the respective sourdoughs. The dominant species in the pre-sourdough phase were *Meyerozyma guilliermondii* for apple sourdough and *Hanseniaspora uvarum* for grape sourdough. For yogurt sourdough, *Saccharomyces cerevisiae* was already the dominant yeast in the pre-sourdough phase with a low proportion of *Meyerozyma guilliermondii* and *Wickerhamomyces anomalus*. In the three sourdoughs, regardless of the different ingredients used, *Saccharomyces cerevisiae* was the dominant yeast in the ripe phase.

Keywords: Sourdough, Apple, Grape, Yogurt, Molecular identification, Yeast

INTRODUCTION

When bakers, whether industrial or artisan, set about making sourdough bread it is because they wish to achieve several objectives. Among others, bakers want to make a type of bread with enhanced acidity, and a distinctive taste and flavour. Sourdough is distinguished from other bread ferments, because the microbiota is composed of a population of both lactic acid bacteria (LAB) and yeast. The isolation and application of molecular techniques for the identification of these microorganisms enables us to improve our knowledge of this singular and complex ecosystem. Regional Spanish bakers value keeping the sourdough fermented for years, without using an initial starter. They only prepare it again when they consider that a sensory quality deviation is produced. Nowadays, there is a trend to prepare the liquid ferment, blocking its activity by applying lower temperatures to decrease the refreshments frequency, instead of maintaining solid ferment with several daily refreshments. Some bakers maintain several sourdoughs at the same time, initiated with special ingredients, or using different types of cereal flours. Such applications are aimed to bread products, with variable proportions of ferment, and some sweet elaboration as the panettone.
Sourdough can be initiated spontaneously using different ingredients, but cereal flour (most frequently wheat or rye) and water are common to every formulation. In all protocols, the subsequent phases involve trying to achieve a stable ripe sourdough (mother dough). After the initial preparation and prefermentation of the sourdough, the refreshment technique is aimed at maintaining the metabolic activity of the microbial communities at all times. Generally, a ripe sourdough contains a variable amount of lactic acid bacteria and yeast, ranging from 10^7 to 10^9 CFU/g and 10^5 to 10^7 CFU/g, respectively, with a ratio of about 100:1 **(Gobbetti, 1998)**. The final pH, which ranges from 3.5 to 4.3, is usually considered an index of well-developed sourdough fermentation **(Collar *et al.*, 1994)**.
LAB comprise a large heterogeneous group of Gram-positive, non-sporulating, and strictly fermentative lactic acid-producing bacteria. It is a group that includes a broad number of genera and species. Although the LAB sourdough microbiota is clearly dominated by *Lactobacillus*, other less predominant or subdominant LAB species may also be found, including members of the genera *Weissella*, *Pediococcus*, *Leuconostoc*, *Lactococcus*, *Enterococcus*, and *Streptococcus* **(De Vuyst and Neysens 2005; Huys *et al.*, 2013)**.
Yeasts are unicellular fungi, and in sourdough various different genera have been detected. A recent review **(Huys *et al.*, 2013)** based on some 40 original publications — from the early 1970s, and from different, mainly European, countries (particularly Italy) — listed *Saccharomyces cerevisiae*, *Candida humilis* (syn. *Candida milleri)*, *Pichia kudriavzevii*, *Kazachstania exigua* (syn. *S. exiguus)*, *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* (syn. *P. anomala*, *Hansenula anomala)* as the six most frequently encountered species; other species are less frequently detected in sourdough, namely *Candida glabrata*, *Pichia membranifaciens*, *Candida parapsilosis*, *Candida tropicalis*, *Candida stellate*, *Kazachstania unispora*, *Kluyveromyces marxianus*, *Meyerozyma guilliermondii* and *Saccharomyces pastorianus*.
Yeasts contribute, more than LAB, to the leavening capability of the dough by releasing CO_2 from alcoholic fermentation. The yeast leavening capacity varies between species and strains and is affected by the LAB metabolism and bread fermentation process conditions **(Häggman and Salovaara, 2008a,b)**. Sourdough leavened products, compared to purely yeast leavened products, increase the shelf life **(Galle and Arendt, 2014)**, and have significant impact on nutritional properties, especially when whole flour is employed **(Poutanen *et al.*, 2009)**. Another important contribution of sourdough yeast, as well as LAB, is to produce different volatile compounds that improve and increase the sourdough taste and flavour. These compounds vary in kind, concentration, and according to strain **(Damiani *et al.*, 1996)**.
The aims of this study were: i) to identify and describe the collection of yeast strains from three spontaneous laboratory sourdoughs prepared using a traditional Spanish protocol, each one elaborated with different initial ingredients, such as apples (5 days juice fermented at 40ºC), yogurt, and grapes (2 days must fermented at 20ºC), and ii) to evaluate the role of these ingredients in the diversity of microbiota yeast sourdough. The use of apple and yogurt is common in Spanish bakeries; nevertheless that of grape is a more recent development.

MATERIAL AND METHODS

Origin of sourdoughs yeast collection

The collection of sourdoughs yeast to be identified was composed of a total of 118 strains. These yeasts came from the three types of spontaneous laboratory sourdoughs prepared, in duplicate, using a traditional Spanish multiphase protocol provided by an artisan bakery ('Fleca Parés', Vilafranca del Penedès, Catalonia, Spain). Each sourdough was started in a sterile container and then, they were prepared with a spiral kneader; the kneading shaft was cleaning meticulously and treated with water at 85°C. Sourdoughs were elaborated with wheat flour (*Triticum aestivum*) and using different initial ingredients such as organic apples, plain yogurt, and organic grapes as described by **Gordún *et al.* (2015)**.

The first sourdough phase was the preparation of the different ingredients (fermented apple juice and lightly fermented grape must). For apple sourdough (AS) small pieces of apple were fermented with unpasteurized honey and mineral water (ratio of 1:0.04:1, respectively) for 5 days at 40°C; and for grape sourdough (GS) grape must (Xarel·lo white grape variety from the Valldolina vineyard) was fermented for 2 days at 20ºC; this first phase was not performed for yogurt sourdough (YS).

The second or pre-sourdough phase lasted for 5 days, for all three sourdoughs. This phase begins with the mixture of the specific ingredients and wholemeal flour (ratio of 3:1, respectively), leaving 48 hours at 22ºC to carry out the mixture fermentation. Later, three wheat flour and water renewals were carried out, in which fermentation and activity blocking periods were interspersed. Specifically, a part of the preceding dough was mixed with flour and mineral water (ratio of 1:5:6, respectively) and left for 24hours at 22 °C; two more renewals were carried out, again mixing a part of the previous dough with flour and mineral water (ratio of 1:1.7:1.7, and 1:2.5:2.5, respectively) and both renewals they were left 2 hours at 22ºC and 22 hours at 5ºC.

Later, throughout the third or ripe sourdough phase, the refreshment was done three times a day (at 9am, 1pm, and 5pm) for 5 days. Specifically for this, a part of the preceding sourdough was mixed with wheat flour and mineral water (ratio of 2:2:1, respectively), leaving 4 hours at 29ºC in the first two refreshments (at 9am and 1pm) and in the third refreshment (5pm) (ratio of 1:2:1, respectively) 16 hours at 29ºC.

Samples were taken throughout the sourdough process; in the first phase from apple juice after 5 day fermentation and grape must after 2 day fermentation. Four samples of the second phase (named P1, P2, P3, P4) and five samples of the third phase at 9am (named R1, R2, R3, R4, R5) were analyzed. The sampling was done immediately after the fermentation period and before the renewal of the ingredients, with the exception of P1 that was analyzed after mixing the specific ingredients with the flour.

As described by **Gordún *et al.* (2015)**, for the counting and isolation of the yeasts from the different phases of three sourdoughs (AS, GS, and YS), 10 g of each sourdough sample was homogenized with 90 mL of saline Ringer ¼ solution (Scharlau, Spain) using a Stomacher apparatus (BagMixer® 400P, Interscience, Paris) for 2 min. Decimal dilutions were then made using the same solution and microbiological seeding was performed in duplicate on every agar plate: i) total yeast count on WL agar supplemented by 0.5 g/L chloramphenicol (Scharlau, Spain), and ii) non-*Saccharomyces* yeast on lysine agar (Scharlau, Spain). The yeast plates were incubated for 3 to 5 days at 27°C. After counting, yeast strains were selected based on their morphology or colony colour in order to represent the diversity of yeast sourdoughs. The yeasts were purified on Sabouraud agar (Scharlau, Spain) and stored at 4°C until identified.

Yeast identification by 5.8S-ITS rRNA analysis and sequencing

Yeast isolates were clustered by amplification and restriction of an rRNA gene region and representative isolates were identified to the species level by sequencing the 5.8S-ITS region. All yeast strains were incubated in YPD broth (2% glucose, 1 % yeast extract, 2% peptone w/v) at 28ºC for 48 hours and cells were collected by centrifugation. DNA was isolated according to **Querol *et al.* (1992)** and diluted to 1-50 ng/µl. The rRNA gene region was amplified in a Mastercycler gradient (Eppendorf). Primer pairs used to amplify the 5.8S-ITS region: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') have been described elsewhere **(White *et al.*, 1990)**. PCR reactions were as follows: a first step at 95ºC for 5 min, then 40 cycles of 94ºC for 1 min, 55.5ºC for 2 min, and 72ºC for 2 min, with a final extension of 10 min at 72ºC. PCR products were digested without further purification by the endonuclease *Hinf*I (New England Biolabs, Inc., Germany) according to the supplier's instructions. Additionally, endonuclease Dde I and Mbo I (New England Biolabs, Inc. Germany) were used to better distinguish among *Hanseniaspora* species and *Candida* species, respectively. The PCR products and their restriction fragments were separated on 1.4 and 3% agarose gels, respectively, in 1x TBE buffer, stained with ethidium bromide and photographed under UV light. Fragment sizes were estimated by comparison against a 100-pb DNA ladder (Roche Diagnostics GmbH, Mannheim, Germany).

Yeast isolates were grouped according their amplicon sizes and restriction fragment length polymorphism. One strain of each group was chosen for sequencing. PCR products of selected strains were cleaned with a QIAquick Gel extraction kit (Qiagen, Germany) and directly sequenced using the Bid Dye Terminator v 3.1 sequencing standard kit (Applied Biosystems) following the manufacturer's instructions, in an automatic sequencer 3730 DNA analyser (Applied Biosystems**).** The amplicons were used as template for the sequencing reaction (BigDye, Qiagen, Germany), and the DNA fragments were sequenced (Secugen, Madrid, Spain). To sequence both strands, ITS1 and ITS4 primers were used. Sequences from the 5.8S-ITS region were aligned using the Vector NTI software (Life Technologies). Sequence comparisons were performed online using the basic local alignment search tool (BLAST) program **(Altschul *et al.*, 1990)** at the National Center for Biotechnology Information **(National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD)**. Strains were ascribed to the species based on comparisons with type strain sequences in (http://www.ncbi.nlm.nih.gov/blast).

RESULTS AND DISCUSSION

Identification of yeast strains isolated from sourdoughs

A total of 118 yeast isolates were clustered using PCR-RFLP analysis of the 5.8S-ITS rRNA region into eight groups. After analysis of their restriction fragment length polymorphism (Table 1) two of these groups — group: *Hanseniaspora uvarum*, and group: *Saccharomyces cerevisiae* — were identified at genus and species level respectively, by comparison of the size of restriction bands with those available in the literature **(Cordero-Bueso *et al.*, 2011; Esteve-Zarzoso *et al.*, 1999)**. The other six groups could not be identified by RFLP analysis. In order to recognize and confirm the species identification, especially when the 5.8S-ITS pattern had not been previously reported, one strain representing each group was selected for sequencing the 5.8S-ITS rRNA amplicon. Sequencing data revealed that the other yeast groups corresponded to *Candida oleophila*, *Candida pararugosa*, *Cyberlindnera misumaiensis*, *Cystobasidium pinicola* (syn. *Rhodotorula pinicola*), *Meyerozyma guilliermondii*, and *Wickerhamomyces anomalus* respectively, and confirmed the previous yeast identification (groups *H. uvarum and S. cerevisiae*).

Table 1 Identification of sourdough yeast isolates by means of amplification of 5.8S-ITS region and sequencing

Species	Strains analyzed	ITS-amplified product	Restriction fragments (*Hinf* I)	Identification*	Isolation source
Candida oleophila	2	630	320 +320	JX188107.1 (100 %)	AS (1)
Candida pararugosa	2	430	220 + 210	GQ458032.1 (99%)	AS (1)
Cyberlindnera misumaiensis	2	610	305 + 305	KY103071.1 (100%)	AS (1)
Cystobasidium pinicola	2	580	210 + 185 + 160	HG532085.1 (99%)	AS (1)
Hanseniaspora uvarum	20	760	360 + 200 + 180	KT029774.1 (99 %)	GS (1,2,3)
Meyerozyma guilliermondii	30	620	320 + 290	JX188191.1 (100%)	AS(1,2,3) YS (2,3)
Saccharomyces cerevisiae	40	880	365 + 365 + 150	KT726921.1 (99%)	AS (2,3) YS (2,3) GS (2,3)
Wickerhamomyces anomalus	18	625	315 + 315	JX188245.1 (99%)	YS (2,3)

Legend: Size in bp of the PCR products and the restriction fragments obtained with endonuclease *Hinf* I of the species identified in this study. Isolation source from sourdoughs produced from apple (AS), yogurt (YS) or grape (GS) during the phases: phase (1) of different ingredients preparation; phase (2) of pre-sourdough; and phase (3) of ripe sourdough. * GenBank Accession (Maximum identity, %)

Results from yeast sourdoughs are presented in Table 1. Thus, the different yeast groups were distributed specifically throughout the development of three types of sourdough prepared with different ingredients, such as apple, yogurt and grape. The results show that four of eight yeast species identified, *C. oleophila*, *C. pararugosa*, *Cyb. misumaiensis* and *Cys. pinicola*, would not form part of these sourdoughs because they were only identified in the apple ingredient, and not in fermented apple juice, which was used in the sourdough (Table 2). Only *S. cerevisiae* was present in all three types of the sourdough studied, including pre-sourdough and the ripe sourdough phase. The other yeast species identified, *H. uvarum, M. guilliermondii* and *W. anomalus*, were mostly associated with a particular sourdough and its predominance varied with the phase of the sourdough.

Other laboratory sourdoughs elaborated with durum wheat flour and with additional ingredients, as macerated pears, concentrated grape must, or unpasteurized honey, showed different strains of *S. cerevisiae* as dominant yeast microbiota in all mature sourdoughs **(Minervini *et al*., 2016)**.

Enumeration and yeast identification from apple sourdough

Forty-two strains from the 3 phases of AS elaboration were isolated, purified and identified (Table 2). In the first phase, the apple juice (small apple pieces in water and honey) initially contained various yeast species with a total population level of 1.9E+03 CFU/g. The species isolated from apple juice were identified as *C. oleophila, C. pararugosa, Cyb. misumaiensis, Cys. pinicola* and *M. guilliermondii. C. oleophila* and *Cyb. misumaiensis* have been identified on cider must **(Coton *et al*., 2006)**; *Cys. pinicola* has been reported in skin of fruits **(Janisiewicz *et al*., 2010)** and *C. pararugosa* in fruit-based beverages such as red wine **(Jensen *et al*., 2009)**. These four species of yeast were not able to adapt to the sourdough ecosystem. In the fermented apple juice ready to be used as a liquid starter to initiate the AS, *M. guilliermondii* was found at a population level of 5.2E+06 CFU/g. During the second phase (5 days for sourdough preparation with the aim of achieving a stable microorganism population as well as adequate pH and acidity), *M. guilliermondii* continued to be the dominant species, with final population levels of 7.6E+05 CFU/g. In the third AS phase, where ripe sourdough is propagated by refreshments (three times a day), a lowered *M. guilliermondii* population was observed reaching levels as low as 2.0E+03 CFU/g, at the same time *Saccharomyces cerevisiae* was beginning to implant, with a population of 9.5E+05 CFU/g.

M. guilliermondii — which was isolated from the beginning to the end of the apple sourdough study — has been previously identified on several varieties of apples, even remaining on the surface after washing **(Pelliccia *et al*., 2011)**. A wide variety of yeast species, including *Saccharomyces* and non-*Saccharomyces*, was found throughout the cider-making process. In general, the non-*Saccharomyces* yeasts grow well during the early stages of apple juice fermentation, but are subsequently replaced during the following stages by *Saccharomyces* yeast **(Suárez *et al*., 2007)**.

M. guillermondii was only occasionally isolated from sourdough, in particular from a Spanish laboratory-made wheat sourdough where it was found, along with *Saccharomyces*, to be the dominant species **(Barber and Baguena, 1988)**. In some tests of bread prepared with both selected *M. guillermondii* strains and baker's yeast, the suitability of *M. guilliermondii* was demonstrated in a mixed starter for extending the shelf-life of sourdough baked goods whilst also maintaining optimal taste and structure **(Coda *et al*., 2013)**.

Enumeration and yeast identification from yogurt sourdough

Forty-four strains from the 2 phases of YS elaboration were isolated, purified, and identified (Table 3). When the initial ingredients — wheat flour, yogurt and water — were mixed, three yeast species were identified, *M. guilliermondii, S. cerevisiae* and *W. anomalus*, at a total population level of 7.0E+01 CFU/g. During the pre-sourdough phase, the population of *S. cerevisiae* increased rapidly reaching 1.7E+07 CFU/g; the other yeast species present at the beginning maintained levels of 8.1E+02 CFU/g. In the ripe sourdough phase, YS could be considered a stable sourdough, in which *S. cerevisiae* maintained a high population level (3.6E+06 CFU/g) and only one secondary yeast, *W. anomalus*, was detected (1.0E+02 CFU/g). Evaluating this YS result, it could be observed, as expected, that the plain yogurt (maintained refrigerated at 5ºC) does not contribute to increase yeast microbiota. When yogurts are produced under conditions of good manufacturing practice, they should contain less than 1 yeast cells/g **(Fleet, 1990)**. The sourdough yeasts may be assumed to have originated from the wheat flour or environmental sources.

W. anomalus has been associated with substrates such as cereals and flours. It is well-adapted to the sourdough microbial ecosystem, where it is encountered either as the only yeast or in an inter-yeast species community **(Daniel *et al*., 2011)**. The dominant species in the Belgian artisan bakery sourdoughs were *S. cerevisiae* and *W. anomalus;* while the dominant species in laboratory sourdough fermented for 10 days were *W. anomalus* and *Candida glabrata* **(Vrancken *et al*., 2010)**. In a pilot plant study made by **Coda *et al*. (2013)**, different combinations of starters previously selected for antifungal activity were tried, and the combination of baker's yeast with *M. guilliermondii*, *W.anomalus* and *Lactobacillus plantarum* showed a more prolonged shelf life and optimal chemical and sensory characteristics compared with the other breads. *W. anomalu*s has been reviewed for its mycocine activities and potential future applications **(Passoth *et al*., 2011; Muccilli *et al*., 2012)**.

Enumeration and yeast identification from grape sourdough

Thirty-two strains from the 3 phases of GS elaboration were isolated, purified, and identified (Table 4). In the first phase, *H. uvarum* was the yeast present in the lightly fermented must. During the second phase, *H. uvarum* dominated reaching a population level of 1.3E+08 CFU/g; in this phase of pre-sourdough *S. cerevisiae* was identified but in a lower proportion. In the third or ripe sourdough phase, *S. cerevisiae* overtook *H. uvarum*, which following the refreshment days reduced its population to 1.3E+03 CFU/g. Thus, initial fermented grape provided a specific yeast microbiota, *H. uvarum*, but in ripe sourdough it was not dominant or stable yeast.

Table 2 Yeast composition in apple sourdough (AS) development using a multiphase protocol (**Gordún *et al*., 2015**)

Sample AS sourdough	Species	Counting CFU/g	Frequency (%)	
		Total yeast	Non-Sacch.	Sacch.
(1). Mix apple, honey, and water	*Candida oleophila* *Candida pararugosa* *Cyberlindnera misumaiensis* *Cystobasidium pinicola* *Meyerozyma guilliermondii*	1.9E+03	100	
(1). Apple juice after 5 day fermentation	*Meyerozyma guilliermondii*	5.2E+06	100	
(2). P1	*Meyerozyma guilliermondii*	4.6E+06	100	
(2). P2	*Meyerozyma guilliermondii*	6.9E+06	100	
(2). P3	*Meyerozyma guilliermondii*	1.1E+05	100	
(2). P4	*Meyerozyma guilliermondii* *Saccharomyces cerevisiae*	7.7E+05	98.7	1.3
(3). R1	*Meyerozyma guilliermondii* *Saccharomyces cerevisiae*	2.9E+04	99.3	0.7
(3). R2	*Saccharomyces cerevisiae* *Meyerozyma guilliermondii*	4.5E+05	4.3	95.7
(3). R3	*Saccharomyces cerevisiae* *Meyerozyma guilliermondii*	9.1E+05	2.0	98.0
(3). R4	*Saccharomyces cerevisiae* *Meyerozyma guilliermondii*	9.9E+05	0.2	99.8
(3). R5	*Saccharomyces cerevisiae* *Meyerozyma guilliermondii*	9.5E+05	0.1	99.9

Legend: Phase (1) of apple ingredient preparation; phase (2) of pre-sourdough (P1-P4); and phase (3) of ripe sourdough propagation (R1-R5). Counting of total yeast corresponds to CFU /g of sourdough in WL agar. *Saccharomyces* yeast frequency (%) was calculated from counting the difference between WL agar (total yeast) and Lysine agar (non-*Saccharomyces* yeast).

The role of non-*Saccharomyces* yeast, such as *H. uvarum,* has been investigated in the wine and cider sectors. The use of specific apiculate yeast in grape must fermentations may lead to the production of wines with different biochemical profiles, and the importance of *Hanseniaspora* yeast in mixed starter cultures with *S. cerevisiae* has been emphasized by **Moreira *et al*. (2011)**. *H. uvarum*

could act in the same way in sourdough. *H. uvarum* has been mentioned in a marginal way as secondary yeast, together with *S. cerevisiae* as the dominant species, in an Italian *panettone* sourdough. The presence of *H. uvarum* was justified by the use of a traditional refreshment procedure using grape must **(Restuccia *et al*., 2007)**.

Table 3 Yeast composition in yogurt sourdough (YS) development using a multiphase protocol (**Gordún *et al.*, 2015**)

Sample YS sourdough	Species	Counting CFU/g	Frequency (%)	
		Total yeast	Non- Sacch.	Sacch.
(2). P1	*Saccharomyces cerevisiae* *Meyerozyma guilliermondii** *Wickerhamomyces anomalus**	7.0E+01	21.4*	78.6
(2). P2	*Saccharomyces cerevisiae* *Meyerozyma guilliermondii** *Wickerhamomyces anomalus**	6.4E+06	0.1*	99.9
(2). P3	*Saccharomyces cerevisiae* *Meyerozyma guilliermondii** *Wickerhamomyces anomalus**	3.5E+07	0.03*	99.97
(2). P4	*Saccharomyces cerevisiae* *Meyerozyma guilliermondii** *Wickerhamomyces anomalus**	1.7E+07	0.01*	99.99
(3). R1	*Saccharomyces cerevisiae* *Wickerhamomyces anomalus*	9.5E+06	0.02	99.98
(3). R2	*Saccharomyces cerevisiae* *Wickerhamomyces anomalus*	8.1E+06	0.01	99.99
(3). R3	*Saccharomyces cerevisiae* *Wickerhamomyces anomalus*	8.0E+06	0.01	99.99
(3). R4	*Saccharomyces cerevisiae* *Wickerhamomyces anomalus*	4.9E+06	0.002	99.998
(3). R5	*Saccharomyces cerevisiae* *Wickerhamomyces anomalus*	3.6E+06	0.002	99.998

Legend: Phase (2) of yogurt pre-sourdough (P1-P4); and phase (3) of ripe sourdough propagation (R1-R5). Counting of total yeast corresponds to CFU/g of sourdough in WL agar. *Saccharomyces* yeast frequency (%) was calculated from counting the difference between WL agar (total yeast) and Lysine agar (non-*Saccharomyces* yeast).

Table 4 Yeast composition in grape sourdough (GS) development using a multiphase protocol (**Gordún *et al*., 2015**)

Sample GS sourdough	Species	Counting CFU/g	Frequency (%)	
		Total yeast	Non-Sacch.	Sacch.
(1). Grape must after 2 day fermentation	*Hanseniaspora uvarum*	7.2E+07	100	
(2). P1	*Hanseniaspora uvarum*	6.4E+07	100	
(2). P2	*Hanseniaspora uvarum* *Saccharomyces cerevisiae*	1.5E+08	86.2	13.8
(2). P3	*Hanseniaspora uvarum*	2.0E+06	100	
(2). P4	*Hanseniaspora uvarum*	2.0E+06	100	
(3). R1	*Hanseniaspora uvarum* *Saccharomyces cerevisiae*	4.2E+06	62.2	37.8
(3). R2	*Hanseniaspora uvarum* *Saccharomyces cerevisiae*	1.7E+05	80.6	19.4
(3). R3	*Saccharomyces cerevisiae* *Hanseniaspora uvarum*	8.0E+04	11.2	88.8
(3). R4	*Saccharomyces cerevisiae* *Hanseniaspora uvarum*	7.7E+04	3.2	96.8
(3). R5	*Saccharomyces cerevisiae* *Hanseniaspora uvarum*	7.0E+04	1.8	98.2

Legend: Phase (1) of grape ingredient preparation; phase (2) of pre-sourdough (P1-P4); and phase (3) of ripe sourdough propagation (R1-R5). Counting of total yeast corresponds to CFU /g of sourdough in WL agar. *Saccharomyces* yeast frequency (%) was calculated from counting the difference between WL agar (total yeast) and Lysine agar (non-*Saccharomyces* yeast).

CONCLUSION

Evaluating jointly the results of yeast composition in three sourdoughs prepared using a traditional Spanish protocol, two of them elaborated with usual and non-essential ingredients like fermented apple juice and yogurt, and the third with a novel ingredient such as lightly fermented must grape, it was observed that AS and GS originally presented a specific population of yeast, namely *M. guilliermondii* and *H. uvarum*, respectively. However, in both types of sourdough, *M. guilliermondii* and *H. uvarum* gave way in the ripe sourdough phase, with the successive refreshments, to *S. cerevisiae* as the dominant species. In the case of YS, the initial yeasts possibly came from the flour, and were identified as *S. cerevisiae*, *M. guilliermondii*, and *W. anomalus*. In the YS pre-sourdough phase, *S. cerevisiae* was already dominant, in contrast to AS and GS, and it continued to be dominant in the ripe sourdough. This study indicates that the addition of different non-essential ingredients to the production of sourdough may provide a specific diversity of yeast, especially of the non-*Saccharomyces* group. It would be of interest to study in greater depth the uses of the different ingredients in the final sourdough refreshment with a view to enhancing the effect of these non-*Saccharomyces* yeasts.

Acknowledgments: Thanks to Marc and Gabriel Parés of the centennial artisan bakery *Fleca Parés*, for providing us with, for the initial study, the recipes and sourdough ingredients, and motivating us to carry out this sourdough research.

REFERENCES

Altschul, S.F., Gish, W, Miller, W., Myers, E.W., & Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*, 403-410. http://dx.doi.org/10.1016/s0022-2836(05)80360-2

Barber, S., & Baguena, R. (1988). Microflora of the sourdough of wheat flour bread. V. Isolation, identification and evaluation of functional properties of sourdough's microorganisms. *Revista de Agroquímica y Tecnología de Alimentos, 28*, 67-78.

Coda, R., Rizzello, C.G., Di Cagno, R., Trani, A., Cardinali, G., & Gobbetti, M. (2013). Antifungal activity of *Meyerozyma guilliermondii*: Identification of active compounds synthesized during dough fermentation and their effect on long-term storage of wheat bread. *Food Microbiology 33*, 243-251. http://dx.doi.org/10.1016/j.fm.2012.09.023

Collar, C., Benedito de Barber, C., & Martínez-Anaya, M.A. (1994). Microbial sourdoughs influence acidification properties and bread-making potential of wheat dough. *Journal of Food Science*, *59*(3), 629-633. http://dx.doi.org/10.1111/j.1365-2621.1994.tb05579.x

Cordero-Bueso, G., Arroyo, T., Serrano, A., Tella, J., Aporta, I., Vélez, M.D., & Valero, E. (2011). Influence of the farming system and vine variety on yeast communities associated with grape berries. *International Journal of Food Microbiology*, *145*, 132-139. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.11.040

Coton, E., Coton, M., Levert, D., Caseregola, S., & Sohier, D. (2006). Yeast ecology in French cider and blanck olive natural fermentations. *International Journal of Food Microbiology,108*, 103-135. https://dx.doi.org/10.1016/j.ijfoodmicro.2005.10.016

Damiani, P., Gobbetti, M., Cossignani, L., Corsetti, A., Simonetti, M.S., & Rossi, J. (1996). The sourdough microflora characterization of hetero- and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds produced. *Lebensmittel-Wissenschaft & Technologie, 29*, 63–70. http://dx.doi.org/10.1006/fstl.1996.0009

Daniel, H.M., Moons, M.C., Huret, S., Vrancken, Y., & De Vuyst, L. (2011). *Wickerhamomyces anomalus* in the sourdough microbial ecosystem. *Antonie Van Leeuwenhoek*, *99*, 63–73. http://dx.doi.org/10.1007/s10482-010-9517-2

De Vuyst, L., & Neysens, P. (2005). The sourdough microflora: biodiversity and metabolic interactions. *Trends in Food Science & Technology*, *16*, 43-56. http://dx.doi.org/10.1016/j.tifs.2004.02.012

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., & Querol, A. (1999). Identification of yeasts by RFLP analysis of the 5.8SrRNA gene and the two ribosomal transcribed spacers. *International Journal of Systematic Bacteriology, 49*, 329-337. http://dx.doi.org/10.1099/00207713-49-1-329

Fleet, G.H. (1990). A review, yeast in dairy products. *Journal of Applied Bacteriology*, *68*, 199-211. http://dx.doi.org/10.1111/j.1365-2672.1990.tb02566.x

Galle, S., & Arendt, E.K. (2014). Exopolysaccharides from sourdough lactic acid bacteria. *Critical Reviews in Food Science and Nutrition*, *7*, 891-901. https://dx.doi.org/10.1080/10408398.2011.617474

Gobbetti, M. (1998). The sourdough microflora, interactions of lactic acid bacteria and yeasts. *Food Science and Technology International*, *9*, 267-274. http://dx.doi.org/10.1016/s0924-2244(98)00053-3

Gordún, E., del Valle, L.J., Ginovart, M., & Carbó, R. (2015). Comparison of the microbial dynamics and biochemistry of laboratory sourdoughs prepared with grape, apple and yogurt. *Food Science and Technology International*, *21*, 428-439. http://dx.doi.org/10.1177/1082013214543033

Häggman, M., & Salovaara, H. (2008a). Microbial re-inoculation reveals differences in the leavening power of sourdough yeast strains. *LWT - Food Science and Technology*, *41*,148-154. http://dx.doi.org/10.1016/j.lwt.2007.02.001

Häggman, M., & Salovaara, H. (2008b). Effect of fermentation rate on endogenous leavening of *Candida milleri* in sour rye dough. *Food Research International*, *41*,266-273. http://dx.doi.org/10.1016/j.foodres.2007.12.002

Huys, G., Daniel, H.M., & De Vuyst, L. (2013). Taxonomy and biodiversity of sourdough yeast and lactic acid bacteria. In Gobbetti, M and Gänzle, M (ed) Handbook on sourdough biotechnology. New York: Springer, pp 105-154.

Janisiewicz, W.J., Kurtzman, C.P., & Buyer, J.S. (2010). Yeasts associated with nectarines and their potential for biological control of brown rot. *Yeast 27*, 389-398. http://dx.doi.org/10.1002/yea.1763

Jensen, S., Umiker, N., Arneborg, N., Charles, G., & Edwards, C. (2009). Identification and characterization of *Dekkera bruxellensis*, *Candida pararugosa*, and *Pichia guilliermondii* isolated from commercial red wines. *Food Microbiology, 26*, 915–921. http://dx.doi.org/10.1016/j.fm.2009.06.010

Minervini, F., Celano, G., Lattanzi, A., De Angelis, M., & Gobbetti, M. (2016). Added ingredients affect the microbiota and biochemical characteristics of durum wheat type-I sourdough. *Food Microbiology* 60, 112-123. http://dx.doi.org/10.1016/j.fm.2016.05.016

Moreira, N., Pina, C., Mendes, F., Couto, J.A., Hogg, T., & Vasconcelos, I. (2011). Volatile compounds contribution of *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* during red wine vinifications. *Food Control 22*(5), 662-667. http://dx.doi.org/10.1016/j.foodcont.2010.07.025

Muccilli, S., Wemhoff, S., Restuccia, C., & Meinhardt, F. (2012). Exoglucanase-encoding genes from three *Wickerhamomyces anomalus* killer strains isolated from olive brine. *Yeast, 30*, 33-43. http://dx.doi.org/10.1002/yea.2935

National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda MD. http://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed 23 February 2016.

Passoth, V., Olstorpe, M., & Schnürer, J. (2011). Past, present and future research directions with *Pichia anomala*. *Antonie van Leeuwenhoek 99*, 121-125. http://dx.doi.org/10.1007/s10482-010-9508-3

Pelliccia, C., Antonielli, L., Corte, L., Bagnetti, A., Fatichenti, F., & Cardinali, G. (2011). Preliminary prospection of the yeast biodiversity on apple and pear surfaces from Northern Italy orchards. *Annals of Microbiology 61*, 965-972. http://dx.doi.org/10.1007/s13213-011-0220-y

Poutanen, K., Flander, L., & Katina, K. (2009). Sourdough and cereal fermentation in a nutritional perspective. *Food Microbiology*, *26*, 693–699. http://dx.doi:10.1016/j.fm.2009.07.011

Querol, A., Barrio, E., & Ramón, D. (1992). A comparative study of different methods of yeast strain characterization. *Systematic and Applied Microbiology 15*, 439-446. http://dx.doi.org/10.1016/s0723-2020(11)80219-5

Restuccia, C., Randazzo, C., Pitino, I., Caggia, C., & Fiasconaro, N. (2007). Phenotypic and genotypic characterization of lactic acid bacteria and yeasts from sourdough for panettone production. *Industrie Alimentari XLVI*, 1231-1236.

Suárez, B., Pando, R., Fernández, N., Querol, A., & Rodríguez, R. (2007). Yeast species associated with the spontaneous fermentation of cider. *Food Microbiology*, *24*, 25-31. http://dx.doi.org/10.1016/j.fm.2006.04.001

Vrancken, G., De Vuyst, L., Van der Meulen, R., Huys, G., Vandamme, P., & Daniel, H.M. (2010). Yeast species composition differs between artisan bakery and spontaneous laboratory sourdoughs. *Fems Yeast Research*, *10*, 471-481. http://dx.doi.org/10.1111/j.1567-1364.2010.00621.x

White, T.J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis MA, Gelfand DH, Sninsky JJ and White, T.J .(ed) PCR protocols, A guide to methods and applications. San Diego: Academic Press, pp 315-322. http://dx.doi.org/10.1016/b978-0-12-372180-8.50042-1