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COMBINED APPROACH FOR THE INVESTIGATION OF DOMINANT FERMENTING MICROBIOTA IN TWO TRADITIONAL SOURDOUGHS PRODUCED IN SICILY

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ABSTRACT

In order to explore the community of lactic acid bacteria (LAB) and yeasts present in two major typical Sicilian sourdoughs, seven mature sourdoughs for "Pane Nero di Castelvetrano" (CV1 - CV3 samples) and "Pane di Monreale" (MR1 - MR4 samples) were analysed through a culture-dependent and culture-independent approach. The highest values of microbial counts were revealed in MR1 sourdough. In particular, LAB counts were at about 10^9 CFU/g in media specific for typical sourdough LAB, such as SDB and SFM, while levels of 10^6 CFU/g were registered for yeasts. The total DNA form each sourdough sample was extracted and subjected to a multiplex-PCR in order to recognize the major groups of LAB. Seventy-six LAB with a rod shape, presumptively *Lactobacillus*, were phenotypically grouped and subjected to a genotypic identification by sequencing of the 16S rRNA gene and further confirmed by species-specific PCRs. Yeasts were isolated and identified by a combined genotypic approach consisting of restriction fragment length polymorphism (RFLP) of 5.8S rRNA gene and sequencing of D1/D2 domain of the 26S rRNA gene. The LAB species identified were *Lactobacillus sanfranciscensis*, *Lactobacillus paralimentarius*, *Lactobacillus brevis* and *Lactobacillus coryniformis*. Among yeasts, *Saccharomyces cerevisiae*, *Pichia guiliermondii*, *Pichia segobiensis*, *Rhodotorula acuta* and *Rhodotorula mucilaginosa* were the species hosted in Sicilian sourdough. The multiplex PCR carried out on total DNA of sourdoughs allowed the rapid identification of the majority of sourdough lactobacilli but the culture-dependent methodology was confirmed to be necessary for the detection of the species, such as *Lactobacillus coryniformis* not included in the system.

1. Introduction

Cereals constitute a major source of dietary nutrients throughout the world (Blandino et al., 2003). They contain the macronutrients (proteins, lipids and carbohydrates) and supply important minerals, vitamins and other micronutrients required by humans for growth and maintenance (Topping, 2007). Dry cereals can only be eaten after grinding and mixing with water (Salovaara, 1998). After a while, thanks to the action of several biological agents including bacteria, yeasts and filamentous fungi, which determine the saccharification of starch in the raw materials and affect microbial protein supply (Herrera-Saldana et al., 1990), the mixture results in the formation of a product characterized by sour aroma.

Cereal-based fermented foods are produced all over the world. Some products, such as sourdoughs employed to produce bread and sweet baked goods, are common to many societies and, for this reason, they have been well characterized for their microbial diversity which play a major role in the determination of the final products. However, the majority of cereal fermented products are local, often obtained with raw materials produced within

The ecological composition of traditional and typical food products, although characterised by a limited diffusion, deserve to be investigated. As a matter of fact, unlike industrial foods processed with a few microbial strains able to dominate the fermentations, artisanal niche products with no commercial starters added are source of high biodiversity. Thus, several strains with different aptitudes, including health promoting features, may be found and used to maintain some characteristics over time (Settanni et al., 2013; Alfonzo et al., *in press*). Moreover, a survey of the dominant populations and strains of a given product is of paramount importance for the valorisation of the food itself, since some strains may be used as marker microorganisms to be searched for the authenticity of raw materials, technology of transformation, as well as production area. With this regard, Pane Nero di Castelvetrano and Pane di Monreale restricted areas, and processed following traditional recipes (Ventimiglia et al., 2015). Thus, the resulting products are characterised by a strong typicality, reflecting the usages of the different cities, even though they are located at a distance of a few kilometres from one another.

Sicily is a region of the South Italy were several niche food products link their history to the production area. Among typical products, those manufactured with sourdough technology are gaining more and more interest for their organoleptic characteristics and also for their prolonged shelf-life, compared to baked goods processed with baker's yeast addition. Different sourdough breads are produced throughout this region, the most known are Pagnotta del Dittaino, Pane nero di Castelvetrano, Pane di Lentini and Pane di Piana degli Albanesi (Minervini et al., 2010; Corona et al., 2016).

Furthermore, recent studies of the bacterial community that characterizes Sicilian sourdoughs highlighted the differences between the populations of lactic acid bacteria in sourdough produced in different areas (Ventimiglia et al., 2015).

represent two typical breads of western Sicily, well appreciated throughout the island and also in the continental Italy. Both breads are obtained with sourdough technology employing *Triticum durum* flour. However, the production of Pane Nero di Castelvetrano involves the use of sourdough as a leavening agent and the mixture of two semolina: whole wheat blond sicilian and ancient varieties of local durum wheat (Tumminia) in the ratio of 30% (w/w). Tummina (*Triticum durum* Desf. var. *reichenbachii*) cultivated in a few towns within Valle del Belìce area (western Sicily), which provides the final bread with the typical dark brown appearance (Giancaspro et al., 2016). The microorganisms hosted in raw materials employed in sourdough might be defining for production (Alfonzo et al., 2013). Sourdough is a complex ecosystem characterized mainly by the presence of lactic acid bacteria (LAB) and yeasts (De Vuyst et al., 2014).

For the above reasons, the aims of the present work were to detect, isolate and identify the dominant LAB and yeast population of the sourdoughs used to produce Pane Nero di Castelvetrano and Pane di Monreale by means of a combined approach including classical and culture-independent microbiological techniques.

2. Materials and methods

2.1.Sourdough sample collection and pH measurement

Sourdoughs used in Pane Nero di Castelvetrano and Pane di Monreale production (Table 1) were collected from bakeries located in Castelvetrano (Trapani province) and Monreale (Palermo province). Samples were taken from five bakeries (CV1 and MR1-MR4), one farm house (CV2) and one restaurant (CV3) prior refreshment with new semolina.

The values of pH were determined electrometrically by means of pH-Meter BASIC 20+ (Crison Instrument S.A., Barcelona, Spain).

Table 1. Characteristics of sourdoughs used for Pane Nero di Castelvetrano and Pane di Monreale production.

Sample	Production level	Dough Yield	Baker's yeast (%)	Fermentation time (h)	Fermentation temperature (°C)	pH
CV1	Bakery-artisanal	180	0	5	18-20	3.48 ± 0.03
CV2	Home-made	170	0	8	Ambient	3.79 ± 0.02
CV3	Home-made	175	0	8	Ambient	4.02 ± 0.02
MR1	Bakery-artisanal	230	0.3	6	30	3.37 ± 0.01
MR2	Bakery-artisanal	160	0.5	5	18	4.34 ± 0.02
MR3	Bakery-artisanal	180	0.5	6	18	4.30 ± 0.01
MR4	Bakery-artisanal	180	0	8	16	4.38 ± 0.01

[dough yield (DY) = weight of dough/weight of flour × 100]

2.2.Microbiological analysis

Each sample (25 g) was suspended in Ringer's solution (225 mL) (SigmaeAldrich, Milan, Italy), homogenised in a stomacher (BagMixer 400; Interscience, Saint Nom, France) for 2 min at maximum speed, and then serially diluted.

LAB were counted on two generic agar media commonly used for this bacterial group: de Man-Rogosa-Sharpe (MRS) (Oxoid, Milan, Italy) and M17 (Oxoid) for generic food rod and coccus LAB, respectively; Sour Dough Bacteria (SDB) (Kline and Sugihara, 1971) as specific medium for LAB associated with the sourdough matrices. All media were added with cycloeximide (10 mg/ml), to avoid yeast growth. Petri dishes were anaerobically incubated at 30°C for 48 h. Tota l yeasts were estimated on Yeast Glucose Chloramphenicol (YGC) agar (Liofilchem, Roseto degli Abruzzi, Italy), incubated aerobically at 25°C for 48 h and on Wallerstein laboratory (WL) nutrient agar incubated aerobically at 28°C for 72 h. All microbiological counts were carried out in duplicate.

2.3.Genotypic investigation of sourdough *Lactobacillus*

The composition of lactobacilli in the sourdoughs was first investigated by the approach consisting of the combined multiplex PCR technique described by Settanni et al. (2005). Total DNA of sourdoughs were extracted from 500 mg of pellet (by centrifugation at 10.000 × g for 5 min) of the first dilutions used for the microbial analysis. Extraction was performed with the FastDNA® Pro Soil-Direct Kit (MP Biomedicals, CA, USA) following manufacturer's instructions.

To ascertain the presence of lactobacilli in all sourdoughs, they were first typed by a multiplex PCR assay named Grouping-multiplex PCR. Subsequently, four multiplex PCR assays, named Group1-, Group2-, Group3-, and Group4-multiplex PCRs, were performed in order to discriminate

Lactobacillus representatives of each group at the species level (Settanni et al., 2005).

2.4.Isolation,phenotypic characterization and identification of rod LAB

After growth, colonies of various shape (at least five with identical morphology) Gram-positive [Gregersen KOH method (Gregersen, 1978)] and catalase negative (determined by transferring fresh colonies from a Petri dish to a glass slide and adding 5% H_2O_2) bacteria (presumptive LAB) were randomly picked up from agar plates and transferred to the corresponding broth media. The isolates were purified by successive sub-culturing and stored in glycerol at −80°C until further experimentations.

The rod cell morphology of the isolates was confirmed by observation with an optical microscope (Olympus, BX60). Subsequently, the presumptive rod LAB isolates were subjected to further phenotypic assays and were grouped on the basis of their growth characteristics: growth at 15 and 45°C; acid production from arabinose, ribose, xylose and sucrose; and CO_2 production from glucose. The test for CO_2 production was carried out in Durham's tubes with MRS broth. Positive results for this test indicated a hetero-fermentative metabolism.

A representative percentage of isolates from each phenotypic group was identified genotypically by 16S rRNA sequencing. Genomic DNA for PCR assays was prepared from sourdoughs isolates after their overnight growth in MRS broth at 30°C. Cells were harvested, and DNA was extracted using an InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Crude cell extracts were used as templates for PCR.

PCR reactions were performed as described by Weisburg et al. (1991). PCR products were visualized by UV transillumination. The amplicons corresponding in size to the molecular weight of the 16S rRNA genes were excised and purified using the QIAquick purification kit (Quiagen S.p.a., Milan, Italy). The resulting DNA was sequenced using the same primers employed for the PCR amplifications. The sequences were compared with those available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997) and EzTaxon-e (http://eztaxon-e.ezbiocloud.net/) (Chun et al., 2007) databases.

Lactobacillus brevis, Lactobacillus paralimentarius and *Lactobacillus sanfranciscensis* were verified by the multiplex PCR strategy reported by Settanni et al. (2005).

2.5.Isolation and identification of yeasts

Yeasts were collected from YGC and WL media. At least five colonies per morphology were randomly collected from agar plates, purified to homogeneity after several sub-culturing steps onto WL medium and subjected to genetic characterization. Genomic DNA for PCR assays was extracted as reported for LAB.

In order to perform the identification of yeasts, all isolates were analyzed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were amplified with the primer pair ITS1/ITS4 (Esteve-Zarzoso et al., 1999) by means of T1 Thermocycler (Biometra, Göttingen, Germany) and subsequently the amplicons were digested with the with the restriction endonucleases *Cfo*I, *Hae*III, and *Hinf*I (MBI Fermentas, St. Leon-Rot, Germany) at 37°C for 8 h. ITS amplicons as well as their restriction fragments were analyzed twice on agarose gel using at first 1.5% (w/v) agarose and then 3 % (w/v) agarose in 1 × TBE buffer. Standard DNA ladders were 1kb Plus DNA Ladder (Invitrogen) and GeneRuler 50 pb DNA Ladder (MBI Fermentas, St. Leon-Rot, Germany).

Sequencing of the D1/D2 domain of the large subunit 26S rRNA gene was performed for the representative isolates of each ITS group. Gene amplification was carried out using the primers NL1 and NL4 (Invitrogen, Milan, Italy) as described by Kurtzman and

Robnett 1998. PCR products (600-bp) were purified using the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's instructions and then subjected to sequencing at MWG Biotech AG (Ebersberg, Germany). The sequences obtained were compared with those available in the GenBank/EMBL/DDBJ (http://www.ncbi.nlm.nih.gov) (Altschul et al. 1997).

2.6.Statistical analysis

Statistical analysis of microbiological counts were performed using Statistica software (StatSoft Inc., Tulsa, OK). Data from microbiological data were analysed using a generalised linear model (GLM) that included the effects of samples and media. The means and pairwise comparisons were evaluated with a post-hoc Tukey's test. A P value <0.05 was deemed significant.

3. Results and discussions

Microbiological analysis and pH measurement

The main microbiological characteristics of the sourdoughs collected in western Sicily are reported in Table 2. pH ranged between 3.37 and 4.38 that is reported as the typical range of values for several Italian sourdoughs (Valmorri et al., 2006; Minervini et al., 2015). The highest counts were registered on SDB for all samples and on MRS for five samples, while those on M17 showed the lowest values. The levels of sourdough LAB estimated on SDB were above 10^7 CFU/g for almost all samples except MR1 that showed concentrations of about 10^9 CFU/g. The levels of yeasts registered on WL were above 10^5CFU/g, while those displayed by YGC results were lower of approximately one order of magnitude except for the sample MR1. Yeast/LAB ratio was 1:100 or 1:10, reflecting the typical proportion among lactic and acetic acid characterizing the mature sourdoughs (Corsetti and Settanni 2007). The fermentation temperature, has affected the chemical and microbiological characteristics of sourdoughs. MR1 was fermented at temperature of 30 °C, that is optimal for the growth of several LAB and this explains the very high levels of LAB registered for this sample. On the contrary, the other sourdoughs characterized by lower counts were refreshed for shorter times (almost 6 – 8 h) and fermented at lower temperatures (ambient or 16 – 20°C). This was reflected also in the final pH which was lowest for MR1 samples. For this samples, the acidification velocity was also influenced by the DY = 230 (Banu et al., 2011; Vogelmann and Hertel, 2011) which was higher compared to that of the other samples (DY = 160-180).

3.1.Genotypic investigation of sourdough *Lactobacillus* composition

The multiplex-PCR showed in **Fig. 1** allowed to distinguish the lactobacilli composition of the sourdoughs as a function of the amplicons generated by each group (Settanni et al. 2005, 2006). In particular, all sourdoughs except MR1 showed a band of 280 bp typical for the lattobacilli belonging to group III: *L. brevis*, *Lactobacillus fructivorans, Lactobacillus hilgardii* and *Lactobacillus sanfranciscensis*. The samples CV1, MR2, MR3 and MR4 showed comparable results for the presence of 3 amplicons of ca. 180, 280 and 1100 bp. In this samples, the group II (*Lactobacillus alimentarius*, *Lactobacillus paralimentarius*, *Lactobacillus farciminis*, and *Lactobacillus mindensis*) and IV (*Lactobacillus fermentum*, *Lactobacillus frumenti*, *Lactobacillus panis* and *Lactobacillus pontis*) were also revealed. CV2 and CV3 samples showed merely the amplicon at 280 bp. The first step of Grouping-multiplex PCR enabled the detection of *Lactobacillus* species *in situ* without the need of colony isolation and cultivation. The presence of species of Groups II, III and IV in sourdough samples under study, confirmed the constant presence of LAB commonly found in Italian sourdoughs (Settanni et al., 2005; Minervini et al., 2012; Reale et al., 2011).

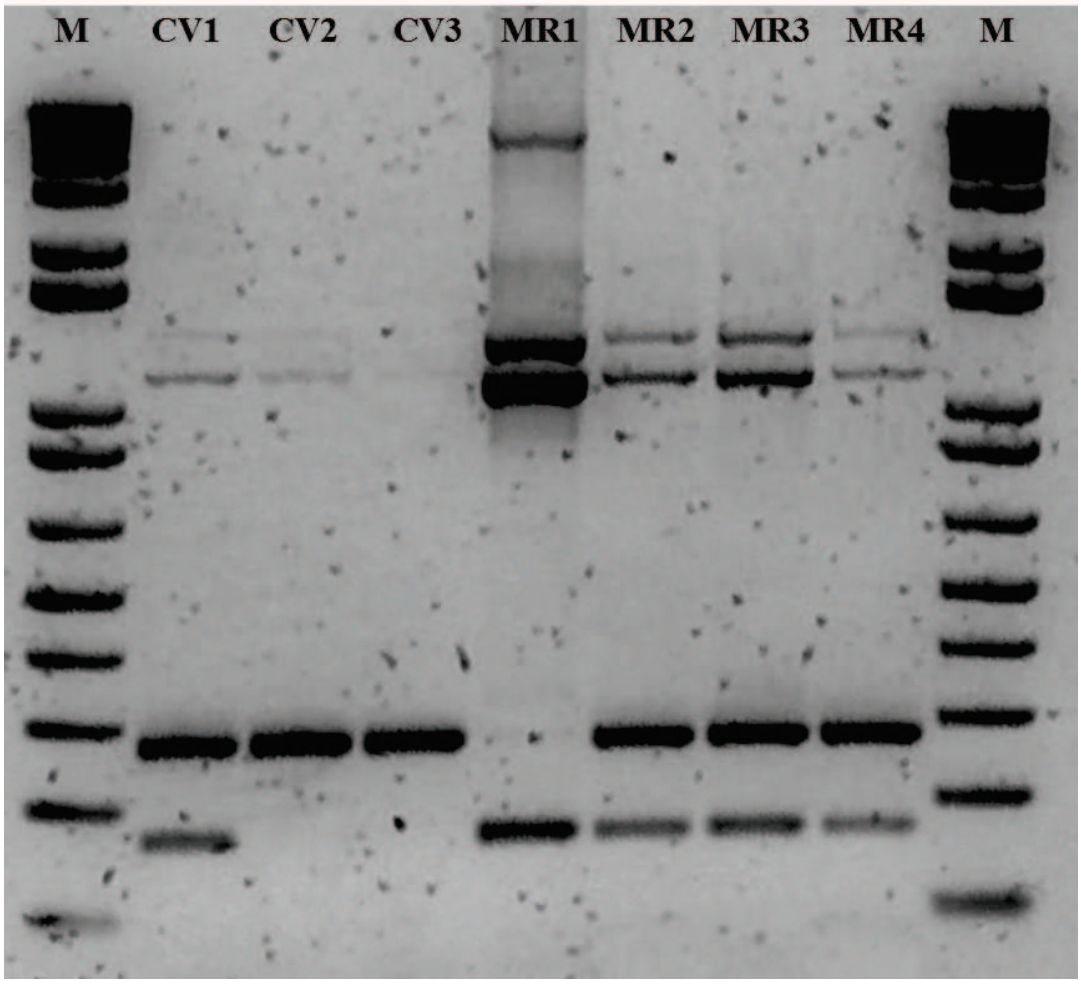

Figure 1. Grouping-multiplex PCR (first step) assay. Abbreviations: M, 1-kb DNA molecular size markers (Invitrogen).

Table 2. Microbiological counts (log CFU/g) of sourdoughs used for Pane Nero di Castelvetrano and Pane di Monreale production.

Sample	LAB				Yeasts	
	MRS	M17	SDB		YGC	WL
CV1	6.90 ± 0.05^{b}	5.21 ± 0.22^{a}	7.61 ± 0.35^{b}		4.42 ± 0.08^{a}	5.58 ± 0.11^{bc}
CV2	5.80 ± 0.14^{a}	4.99 ± 0.13^{a}	7.65 ± 0.17^{b}		4.45 ± 0.08^{a}	5.08 ± 0.25^{c}
CV3	5.63 ± 0.13^{a}	5.00 ± 0.22^{a}	7.69 ± 0.12^{b}		4.18 ± 0.21^{a}	5.19 ± 0.15^{c}
MR1	7.90 ± 0.05^{c}	4.95 ± 0.16^{a}	9.30 ± 0.09^{a}		6.36 ± 0.13^{c}	6.85 ± 0.18^{a}
MR2	6.88 ± 0.05^{b}	6.27 ± 0.20^{b}	7.30 ± 0.21^{b}		5.18 ± 0.32^{b}	5.37 ± 0.20^{c}
MR3	6.84 ± 0.12^{b}	6.01 ± 0.24^{b}	7.57 ± 0.16^{b}		5.45 ± 0.09^{b}	5.88 ± 0.10^{b}
MR4	6.83 ± 0.21^{b}	6.23 ± 0.16^{b}	7.54 ± 0.28^{b}		4.31 ± 0.21^{a}	5.18 ± 0.02^{c}
Statistical significance	***	***	**		**	***

Data within a column followed by the same letter are not significantly different according to Tukey's test.
Abbreviations: MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; M17, medium 17 agar for mesophilic coccus LAB; SDB, sourdough bacteria agar for typical sourdough LAB; YGC, Yeast Extract Glucose Chloramphenicol Agar for yeast; WL, Wallerstein Laboratory Nutrient agar for yeasts.

Table 3. Phenotypic grouping of the rod LAB isolated from sourdoughs used for Pane Nero di Castelvetrano and Pane di Monreale production.

Characters	Clusters			
	A (n=32)	B (n=26)	C (n=10)	D (n=8)
Growth:				
15°C	+	+	+	+
45°C	-	-	-	-
Acid production from:				
arabinose	-	+	-	-
ribose	-	+	+	-
xylose	-	+	-	-
sucrose	-	+	+	+
CO_2 from glucose	+	+	+	-

3.2.Isolation, phenotypic characterization and identification of rod LAB

A total of 209 colonies were collected from the seven sourdoughs used for Pane Nero di Castelvetrano and Pane di Monreale production. All cultures were microscopically inspected and, after Gram determination and catalase test, 76 isolates were classified as presumptive lactobacilli. Based on the combination of the phenotypic features evaluated, the 76 LAB cultures were separated into 4 groups (Table 3). The largest groups were groups A and B (32 and 26 isolates, respectively). All groups included mesophilic lactobacilli growing at 15°C but not at 45°C. Groups A and D were characterized by an obligately hetero-fermentative metabolism showing the ability to produce CO_2 from glucose.The isolates representative of each phenotypic group, for all sourdoughs, were also identified by sequencing of the 16S rRNA gene. The sequences were compared with those available in two distinct databases; all isolates processed were clearly identified as rod LAB, since sequence similarity was higher than 97% in both databases (Table 4) and all species belonged to the genus *Lactobacillus*. The species detected were *L. sanfranciscensis* (n=10), *L. brevis* (n=6), *L. paralimentarius* (n=3) and *L. coryniformis* (n=3). All the species identified are commonly associated with wheat, wheat flour and bakery products (Corsetti et al., 2007; Alfonzo et al., 2013; Ventimiglia et al., 2015). The strains of *L. brevis*, *L. sanfranciscensis* and *L. paralimentarius* were also analyzed with species-specific PCR strategies (Settanni et al., 2005). This technique confirmed their identity. For *L. coryniformis* the unequivocal identification was obtained by applying the sequencing of the 16S rRNA gene because the multiplex PCR assay does not include this species. *L. coryniformis* is a species that is commonly dominant in oat and sorghum sourdoughs and exceptionally can be found in sourdoughs made with durum wheat flour (De Vuyst et al., 2005; Hüttner et al., 2010; Sekwati-Monang et al., 2011), but it is commonly found in raw materials (Alfonzo et al., 2013; Alfonzo et al., in press).

Table 4. Identification of rod LAB strains isolated from sourdoughs used for Pane Nero di Castelvetrano and Pane di Monreale production.

Strain	Amplicon Multiplex PCR (bp)	Species	Sourdough Sample	% similarity (accession no. of closest relative) by:		Sequence length (bp)
				GenBank	EzTaxon	
LABCM02	134	*Lactobacillus sanfranciscensis*	CV1	99 (AJ422037.1)	99.51 (X76327)	1,421

LABCM08	502	*Lactobacillus brevis*	CV1	99 (KU315055.1)	99.55 (KI271266)	1,348
LABCM34	521	*Lactobacillus paralimentarius*	CV1	97 (AJ422034.1)	97.25 (BAMH01000179)	1,447
LABCM59	502	*L. brevis*	CV2	99 (KU555383.1)	99.03 (KI271266)	1,343
LABCM71	134	*L. sanfranciscensis*	CV2	99 (KM822616.1)	99.58 (X76327)	1,419
LABCM78	n.d.	*Lactobacillus coryniformis*	CV2	99 (KX430830.1)	99.79 (GL544638)	1,432
LABCM84	502	*L. brevis*	CV3	100 (KF975703.1)	99.66 (KI271266)	1,171
LABCM85	134	*L. sanfranciscensis*	CV3	99 (AJ422037.1)	99.37 (X76327)	1,423
LABCM86	502	*L. brevis*	CV3	99 (KX010095.1)	98.64 (KI271266)	1,253
LABCM99	n.d.	*L. coryniformis*	MR1	97 (KX430830.1)	97.68 (GL544638)	1,340
LABCM109	521	*L. paralimentarius*	MR1	98 (KC755102.1)	97.43 (BAMH01000179)	1,404
LABCM111	134	*L. sanfranciscensis*	MR2	98 (CP002461.1)	98.26 (X76327)	1,329
LABCM113	502	*L. brevis*	MR2	99 (KU315055.1)	99.85 (KI271266)	1,341
LABCM121	134	*L. sanfranciscensis*	MR2	99 (KM822617.1)	98.82 (X76327)	1,440
LABCM130	134	*L. sanfranciscensis*	MR2	99 (CP002461.1)	99.72 (X76327)	1,419
LABCM138	134	*L. sanfranciscensis*	MR3	99 (KM822615.1)	99.55 (X76327)	1,354
LABCM140	134	*L. sanfranciscensis*	MR3	99 (AJ422037.1)	99.64 (X76327)	1,404
LABCM164	502	*L. brevis*	MR3	99 (EU177644.1)	99.86 (KI271266)	1,428
LABCM174	134	*L. sanfranciscensis*	MR4	100 (CP002461.1)	99.78 (X76327)	1,344
LABCM181	521	*L. paralimentarius*	MR4	100 (KC755102.1)	99.93 (BAMH01000179)	1,353
LABCM186	134	*L. sanfranciscensis*	MR4	99 (AJ422037.1)	99.08 (X76327)	1,401
LABCM188	n.d.	*L. coryniformis*	MR4	97 (KX430830.1)	97.05 (GL544638)	1,357

Table 5. Molecular identification and distribution of yeasts isolated in sourdough used for Pane Nero di Castelvetrano and Pane di Monreale production.

Strain	Species	R. P.	5.8S-ITS PCR	Size of restriction fragments			% similarity[a] (accession no. of closest relative) by:	Isolation source
				*Cfo*I	*Hae*III	*Hinf*I		
YCM125	*P. guilliermondii*	I	650	300+265+60	400+115+90	320+300	99 (EU807915)	MR3
YCM140	*P. segobiensis*	II	640	300+285	490+140	310+310	99 (DQ409151.1)	CV1, CV2, CV3
YCM120	*R. acuta*	III	670	355+315	650	250+190+185	99 (KP216512.1)	MR3
YCM156	*R.mucilaginosa*	IV	630	320+240+80	425+215	340+225+75	99 (AF335986.1)	CV2
YCM02	*S. cerevisiae*	V	850	360+340+140 +50	320+225+165+135	385+125+50	99 (GU138462.1)	MR1, MR2, MR3, MR4

Abbreviation: R.P., restriction profile.
All values for the 5.8S-ITS PCR, 26S PCR and restriction fragments are given in bp.
[a] According to BlastN search of D1/D2 26S rRNA gene sequences in NCBI database.

3.3.Isolation and identification of yeasts

A total of 101 colonies of yeasts were isolated from count plates. They were purified to homogeneity and grouped on the basis of colony appearance on WL medium. The yeasts representative of each morphology were subjected to the molecular identification. The results of the restriction analysis of 5.8S-ITS region were used to cluster the isolates into five groups (Table 5). The sequencing of D1/D2 domain of the 26S rRNA gene identified five species: *Pichia guilliermondii* (group I), *Pichia segobiensis* (group II), *Rhodotorula acuta* (group III), *Rhodotorula mucilaginosa* (group IV), *Saccharomyces cerevisiae* (group V).

The majority of yeasts belonged to the species *S. cerevisiae* and *Pichia segobiensis*. In particular, *S. cerevisiae* were found in all sourdoughs used for Pane di Monreale production while, *P. segobiensis* in all sourdoughs used for Pane Nero di Castelvetrano production. The presence of *S.*

cerevisiae might be due to cross-contamination by conventional bread production (Vrancken et al., 2010). *Rhodotorula acuta* and *P. guilliermondii* were both found only in the sample MR3 while, *R. mucilaginosa* in the sample CV2. Among these yeasts only *S. cerevisiae* was previously isolated from Sicilian sourdoughs (Pulvirenti et al., 2001; Giannone et al., 2010) while, *R. mucilaginosa* from traditional sourdoughs collected from western region in Inner Mongolia of China (Zhang et al., 2011) and *P. guilliermondii* from French and Turkish sourdoughs (Lhomme et al., 2016; Yağmur et al., 2016). The other two yeast species isolated in the present work are not commonly associated with sourdoughs.

4. Conclusions

Sourdough is a specific and stressful ecosystem inhabited by LAB, mainly hetero-fermentative lactobacilli, and yeasts. The results of this study confirmed the codominance of hetero-fermentative lactobacilli in typical Sicilian sourdoughs and the high levels of *L. sanfranciscensis* that is considered the key sourdough LAB. *S. cerevisiae* dominated the yeast community on certain sourdoughs, probably due to the cross-contamination. Works are being prepared to investigate the technological properties of the dominant strains in order to develop *ad hoc* starter cultures constituting stable elements for the typicality of the Sicilian sourdough productions.

5. References

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