Selection of yeasts for their anti-mold activity and prospective use in table olive fermentation

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Abstract

The growth of molds represents a major problem during table olive fermentation. Molds are recognized as spoilage agents and may reduce the product safety for their ability to produce mycotoxins. Yeasts, instead, are usually found in table olive processing and are generally considered desirable microorganisms for their technological properties. In the present study, a model system to select anti-mold yeasts usable as protective adjunct cultures in table olive fermentation was developed. Two hundred and ninety-nine strains of yeasts, isolated from cheese, olive, vinegar, and wine, were tested in vitro for their growth characteristics, fermentative activity, salt tolerance, and antagonistic activity against 10 mold strains isolated from table olives and olive brines. Experimental steps led to the selection of a strain of *Yarrowia lipolitica* exhibiting all the previously listed characteristics.

Practical applications

A strain of *Yarrowia lipolytica* was selected because of its high potentiality as adjunct culture in table olive fermentation to contrast mold growth and increase the product quality and safety. The experimental model system developed in this study may be easily applied to select yeasts usable as adjunct cultures in table olive fermentation in order to contrast mold growth and increase the product safety. These outcomes showed how the use of selected yeasts constitutes a promising way to control mold growth during table olive fermentation.

1. INTRODUCTION

Table olives are among the most produced fermented vegetables worldwide. They represent an important economic source, especially in the Mediterranean countries. In fact, most table olives are produced in Spain, Turkey, Egypt, Greece, and Italy (Arroyo-López et al., 2012). Mold growth is a major problem in table olive processing (El Adlouni, Tozlovanu, Naman, Faid, & Pfohl-Leszkowicz, 2006; Eltem, 1996; Ghitakou, Koutras, Kanellou, & Markaki, 2006; Leontopoulos, Siafaka, & Markaki, 2003; Tantaoui-Elaraki & Letutour, 1985) and several studies reported the presence of complex mold consortia during table olive fermentations (Arroyo-López et al., 2016; Bavaro et al., 2017). Molds are not only considered spoilage microorganisms that can cause several alterations - such as flesh softening and development of moldy taste - but can also represent a serious hazard for consumers for their capacity to produce mycotoxins (Bavaro et al., 2017). Molds belonging to the genera *Aspergillus* and *Penicillium* have been detected in different olive fermentation processes (Heperkan, Meric, Sismanoglu, Dalkiliç, & Güler, 2006). Particularly, *Penicillium crustosum* is one of the most present molds in fermented table olives and can produce toxic metabolites, such as dehydrocyclopeptin, andrastin A, cyclopenol, penitrem A, roquefortine C, viridicatol (Bavaro et al., 2017), and thomitrems A and E (Rundberget & Wilkins, 2002). Regarding *Aspergillus* spp., several species belonging to the section *Nidulantes* are able to produce aflatoxins, sterigmatocystin, emestrin, fumitremorgins, asteltoxins, and paxillin (Chen et al., 2016). Chemical and physical methods, for example, use of potassium sorbate and sodium benzoate (Turantaş et al., 1999), natamycin (Hondrodimou, Kourkoutas, & Panagou, 2011), washing solutions

(Değirmencioğlu, Gürbüz, Değirmencioğlu, & Yildiz, 2014), and high hydrostatic pressure (HHP) (Argyri, Panagou, Nychas, & Tassou, 2014; Tokuşoğlu, Alpas, & Bozoğlu, 2010), have been applied to reduce mold growth during table olive production. The biological control of molds is a promising alternative to the use of chemical additives. For example, lactic acid bacteria can synthesize several antifungal compounds, such as cyclic dipeptides, phenyl-lactic acid, proteinaceous compounds, and 3-hydroxylated fatty acids (Schnürer & Magnusson, 2005), and their effectiveness as biopreservatives in several foods has been demonstrated (Fernandez et al., 2017; Gerez, Torino, Rollán, & de Valdez, 2009; Kachouri, Ksontini, & Hamdi, 2014). Yeasts are commonly isolated from different foods, including table olives (Bautista-Gallego et al., 2011; Pereira, Ramalhosa, Borges, Pereira, & Baptista, 2015), and several strains were used as biocontrol agents against mold growth in apples, grain, and sorghum (Ädel Druvefors & Schnürer, 2005; Rosa, Tauk-Tornisielo, Rampazzo, & Ceccato-Antonini, 2010; Vero, Mondino, Burgueno, Soubes, & Wisniewski, 2002; Zhang, Spadaro, Garibaldi, & Gullino, 2011). They are considered important microorganisms in table olive fermentation for their technological properties and different beneficial effects for human health (Arroyo-López et al., 2012). However, some yeasts are recognized as undesirable microorganisms because their fermentative, pectolytic and xylanolytic activities can cause softening and gas-pocket spoilage of fruits (Bevilacqua et al., 2015; Vaughn, Stevenson, Davé, & Park, 1972). Therefore, the selection of yeasts and their potential use in table olive processing must consider several factors and could take a long time. The aim of this study was to develop a specific fast model system for the selection in vitro of yeasts for their anti-mold activity usable as adjunct cultures in table olive fermentation.

2. MATERIALS AND METHODS

2.1. Microorganisms

Two hundred and ninety-nine strains of yeasts were sourced from the Collection of the Laboratory of Microbiology (Department AGRARIA, Mediterranea University of Reggio Calabria, Reggio Calabria, Italy). Yeasts were previously isolated from different food matrices (cheese, olive, vinegar, wine) and were identified at the genus level, according to morphological features. All the strains were stored at −80°C using cryopreservative bead storage system Microbank TM (Pro-Lab Diagnostics, Canada). Mold strains were isolated from olives and brines during table olive fermentation. In particular, 10 strains, almost all constituting the prevailing isolated mold of the sample, were molecularly identified and utilized to evaluate the anti-mold activity of the yeasts. The 10 mold strains are listed in Table 1.

2.2. Molecular identification of fungal strains

The 10 mold strains and the best yeast strain were molecularly identified using the internal transcribed spacer region of the rDNA as barcode gene. The ITS1-5.8S-ITS2 region was amplified with primers ITS5 and ITS4 (White, Bruns, Lee, & Taylor, 1990) and sequenced by Sanger capillary electrophoresis. Obtained sequences were preliminarily analyzed by BLAST, to identify the genus, and subsequently phylogenetically analyzed along with validated reference sequences available from specific studies of the involved taxonomic groups. Analyses were performed as described by Schena et al. (2014).

2.3. Characterization of yeast strains

2.3.1. Growth characteristics and fermentative activity

A bead of each yeast strain stored at −80°C was inoculated in a test tube containing 10 ml sterile yeast extract peptone dextrose (YPD) broth and one Durham tube—inserted upside down—used to detect production of gas by yeasts. After incubation at 25°C for 5 days, gas production was evaluated based on the presence/absence of bubbles in the Durham tubes and gas-producing strains were excluded. In addition, in order to valorize the ability to grow at the top of the liquid medium—so competing more efficaciously with molds—yeasts able to form an evident layer on the surface of YPD broth were selected for the subsequent phases.

2.3.2. Salt tolerance

Yeasts selected in the previous steps were inoculated in 10 ml tubes of YPD broth containing 7% NaCl and incubated at 25°C for 5 days. The growth ability of strains in the presence of 7% NaCl was assessed observing the presence of an evident layer on the surface of the medium.

2.4. Antagonistic activity of yeasts

The antagonistic activity was evaluated for eight yeasts selected in previous steps because they were no-gas producers, able to form a conspicuous layer on the surface of YPD broth and able to grow in the presence of 7% NaCl. These yeasts were tested for their antagonistic activity against the ten mold strains listed in Table 1. In a first trial (Test 1), strains were precultured in YPD broth (yeasts) and YPD agar (molds) at 25°C for 72 hr. A spore suspension of each mold was prepared from YPD agar plates adding 2 ml sterile physiological solution on the agar surface and scraping it with an L-shaped spreader. Obtained solutions were filtered and used to impregnate sterile paper disks. Therefore, 0.1 ml of each yeast preculture was distributed in new YPD agar plates using an L-shaped spreader until the inoculum was completely absorbed and the antimicrobial susceptibility test disks (Oxoid Ltd, United Kingdom) impregnated with the spore suspensions were placed on the agar surface (four disks for each plate). Plates were incubated at 25°C for 8 days. The diameters of growth of each mold were measured and expressed in mm. Mean value of all growth diameters was calculated in order to evaluate the presumed antagonistic activity of yeasts. In a second trial (Test 2), yeast precultured as described before were propagated on the surface of YPD agar plates. When plates were completely dried, 20 µl spore suspension of each mold were used to create the spots (four spots for plate). Plates were incubated and analyzed as described before.

3. RESULTS

3.1. Molecular identification of fungal strains

According to ITS sequences, fungal isolates obtained from olive drupes and brine were identified as *P. crustosum* (Visagie et al., 2014), *Aspergillus sect. Nidulantes* (Samson et al., 2014), *Malassezia restricta* (Castellá, Dall'Acqua Coutinho, & Cabañes, 2014), and *Galactomyces candidum* (Vu et al., 2016). The list of fungal taxa and relative GenBank accession numbers are reported in Table 1. The analysis of the ITS sequence of the anti-mold yeast strain (GenBank Accession No. MN007127) enabled its identification as *Yarrowia lipolytica* (Vu et al., 2016).

3.2. Evaluation of growth characteristics

Only 180 of the 299 yeast strains showed the required growth ability. In fact, they were able to form a conspicuous layer on the surface of the liquid medium and were selected for the next phase.

3.3. Evaluation of fermentative activity

Only 8 of the remaining 180 yeast strains exhibited no gas production in YPD broth + Durham tube. Consequently, they were selected for the next trial.

3.4. Evaluation of salt tolerance

All the eight yeasts tested showed an excellent capacity to growth in YPD + 7% NaCl. After 5 days at 25°C, the turbidity increased and an evident layer of yeasts was detected in all tubes.

3.5. Antagonistic activity assay

Outcomes on the evaluation of antagonistic activity of yeasts are reported in Table 2 (Test 1) and in Table 3 (Test 2). In Test 1, the strain L352 showed the best performance. Indeed, it was able to inhibit the growth of all molds tested. The strain L908 inhibited the growth of six molds and showed overall a good antagonistic activity (mean value of mold growth diameters = 8.78 mm), followed by strain L874 (mean value of

mold growth diameters = 8.83 mm) and strain L1163 (mean value of mold growth diameters = 11.20 mm). Yeasts L1367, L1371, L1369, and L1372 showed the worst performances (mean values of mold growth diameters from 25.73 to 33.79 mm). Test 2 confirmed the antagonistic behavior of yeasts detected in Test 1. Moreover, in this trial, the strain L352 inhibited the growth of all molds. Strain L874 was able to inhibit the growth of three molds and showed a good antagonistic activity against the others (mean value of mold growth diameters = 18.16 mm), followed by the strains L1163 (mean value of mold growth diameters = 18.33 mm) and L908 (mean value of mold growth diameters = 24.68 mm). Yeasts L1369, L1372, L1371, and L1367 confirmed their limited antagonistic activity (mean values of mold growth diameters from 34.73 to 37.45 mm). Therefore, the strain L352, identified as *Y. lipolytica*, was the yeast with the widest antagonistic activity.

4. DISCUSSION

The experimental method applied in our study permitted to complete the screening of 299 yeasts in four steps. In the first trial, the aim was to select the yeasts that grew at the top of the liquid medium forming a conspicuous layer because, in table olive fermentation, mold growth often covers the brine surface (Heperkan et al., 2006), so yeasts that can grow on the surface seem to be the most suitable for their potential competitive activity. Afterward, the evaluation of the fermentative activity of yeasts was performed in order to exclude the gas-producing strains. Indeed, an excessive CO2 production during olive fermentation can damage the fruits and cause great economic losses (Arroyo-López et al., 2012; Vaughn et al., 1972). Considering the characteristics of brines, yeasts to be used in table olive fermentation should be able to growth in a high-salt environment. In our study, selected yeasts grew in the presence of 7% NaCl developing a clearly visible layer on the surface of the liquid medium. On the other hand, since Greek-style production system requires a salt concentration until 10% (w/v), it will be possible, if necessary, to study salt tolerance at higher values than 7% NaCl with the same method. Antagonistic activity assay led to the selection of the strain L352 of *Y. lipolytica* able to inhibit the growth of all molds in the two tests performed. *Y. lipolytica* is a yeast often isolated from hydrophobic substrates, rich in alkanes and fats, for example, cheese, yoghurt, soy sauce, meat, and shrimp salad, and is used in many industrial processes to obtain various products, such as citric and isocitric acids, γ-decalactone, lipase, and for its ability to produce and store lipids (Gonçalves, Colen, & Takahashi, 2014). Furthermore, it has been demonstrated that some *Y. lipolytica* strains are able to reduce pollution in olive mill wastewaters (Gonçalves, Lopes, Ferreira, & Belo, 2009). Regarding the antibacterial and the anti-mold action of *Y. lipolytica*, several studies have shown that this microorganism may have antagonistic activity against *Listeria* spp., *Bacillus cereus*, and molds, including *Penicillium roqueforti* (Addis, Fleet, Cox, Kolak, & Leung, 2001; Goerges, Aigner, Silakowski, & Scherer, 2006; Monnet et al., 2010; Van den Tempel & Jakobsen, 2000). In this study, the strain L352 of *Y. lipolytica* was the only one that completely inhibited the growth of all 10 molds in both tests performed. Considering also its excellent technological characteristics, this strain is a promising candidate for the biological control of mold growth during table olive fermentation. On the other hand, it may be important to also consider that the role of the molds in the table olive fermentation process should be also useful, similarly to other food productions, such as sausage surfaces (Bavaro et al., 2017). At present, mold role in table olive production is not completely understood yet. So, it may be useful to perform biochemical characterization for technological and safety traits of the molds, such as safety assessments for the production of biogenic amines and mycotoxins. Selection and production of industrialized mold starter cultures may allow olive producers to reduce the risks for consumer safety and to improve organoleptic traits and taste.

5. CONCLUSIONS

In our opinion, this study constitutes a step to solve the problem of mold growth and the consequent mycotoxins accumulation during olive fermentations, in order to improve shelf life and safety of table olives. The method proposed in this paper allowed to select, in a few steps,

yeasts with anti-mold activity, considering also their technological characteristics, and excluding the strains that could damage the product. The experimental steps led to the selection of the strain L352 of *Y. lipolytica* that will be used as adjunct cultures in table olive fermentation to contrast mold growth.

In view of the growing consumer demand for foods without chemical additives, obtained results show that the use of selected yeasts is a promising way for the biological preservation of food.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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TABLE 1. List of the molds used for the antagonistic activity assay

Strain	**Identification**	**Origin**	**Percentage of presence**	**GenBank Acc. No.**
M1	*Penicillium crustosum*	Table olives	50%	MN017794
M2	*Aspergillus* section *Nidulantes*	Table olives	50%	MN017795
M5	*Penicillium crustosum*	Brine	80%	MN007128
M6	*Penicillium crustosum*	Brine	50%	MN007129
M7	*Penicillium crustosum*	Brine	50%	MN007130
M8	*Malassezia restricta*	Brine	91%	MN007131
M10	*Penicillium crustosum*	Brine	75%	MN007132
M11	*Penicillium crustosum*	Brine	99%	MN007133
M12	*Penicillium crustosum*	Brine	99%	MN007134
M15	*Galactomyces candidum*	Brine	13%	MN007135

TABLE 2. Anti-mold activity of yeasts (Test 1)

Yeasts		Mold growth diameters (mm)										
Strains	Origin	M1	M2	M5	M6	M7	M8	M10	M11	M12	M13	Mean value
L352	Cheese	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
L874	Olive	25.0	0.0	26.0	0.0	15.0	0.0	12.0	0.0	10.3	0.0	8.8
L908	Olive	27.3	0.0	28.0	0.0	21.0	0.0	11.5	0.0	0.0	0.0	8.8
L1163	Olive	24.0	8.0	26.0	7.0	22.0	0.0	10.0	0.0	6.0	9.0	11.2
L1367	Vinegar	28.5	12.0	29.5	19.0	30.0	28.0	29.0	25.3	19.0	37.0	25.7
L1369	Vinegar	29.0	14.0	26.0	15.0	33.0	29.0	28.0	32.0	33.0	39.0	27.8
L1371	Vinegar	28.0	13.0	25.0	15.0	31.3	29.0	29.0	33.5	32.0	40.0	27.6
L1372	Vinegar	28.3	60.0	24.0	16.5	3.0	30.0	28.0	34.0	35.1	49.0	33.8

TABLE 3. Anti-mold activity of yeasts (Test 2)

Yeasts		Mold growth diameters (mm)										
Strains	Origin	M1	M2	M5	M6	M7	M8	M10	M11	M12	M13	Mean value
L352	Cheese	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
L874	Olive	32.3	21.0	26.0	23.5	32.0	23.0	0.0	0.0	23.8	0.0	18.2
L908	Olive	30.0	19.0	25.5	21.0	33.0	27.3	21.0	34.0	36.0	0.0	24.7
L1163	Olive	250.	16.0	26.0	17.0	30.0	0.0	22.0	0.0	29.0	0.0	18.3
L1367	Vinegar	350.	51.0	34.0	28.0	38.0	39.4	31.0	35.1	39.0	44.0	37.4
L1369	Vinegar	33.3	50.0	35.0	27.0	37.0	35.0	31.0	32.0	37.0	30.0	34.7
L1371	Vinegar	32.0	48.0	33.0	26.0	38.0	35.0	30.0	30.0	36.0	41.0	34.9
L1372	Vinegar	31.0	45.0	32.0	29.0	36.4	34.0	30.0	31.0	37.0	43.0	34.5