Effect of brine composition on yeast biota associated with naturally fermented *Nocellara messinese* table olives

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ABSTRACT

The study aims to assess the yeast biota associated with table olives of the cultivar *Nocellara messinese* naturally fermented using different brines. Microbial counts, physico-chemical parameters, and yeast consortia was evaluated at days 0, 3, 7, 15, 30, 60, 90, 180, and 240. Yeasts were identified by PCR-RFLP of the 5.8S ITS rRNA region and sequencing of D1/D2 domain of the 26S rRNA gene. At the fermentation end, the yeast population was assessed by PCR-DGGE analysis. Throughout the eight months of fermentation, the brine modification determined different evolution of the yeast species. The salt concentration shift from 5% to 8% delayed the growth of some species at the end of the fermentation. The experimental brine acidification determined a growth of *Pichia mexicana* and *Saccharomyces cerevisiae* that were not found in the other brine formulations. *Pichia kudriavzevii* was the most isolated species followed by *Wickeramomyces anomalus, Zygoascus hellenicus, Candida boidinii*, *Saccharomyces cerevisiae, Candida aaseri*, *Candida tropicalis*, *Candida diddensiae*, *Zygoascus meyerae*, and *Pichia mexicana*. *P. kudriavzevii* and *W. anomalus* species were present in all the processed olives. Some of the strains exhibited suitable technological properties so they could be good candidates as starter in table olive fermentations.

Keywords: yeasts, *Nocellara messinese*, brine formulations, molecular identification, technological properties

1. Introduction

Table olives are among the most appreciated fermented foods in the Mediterranean countries. Greek-style or natural fermentation involves the olives placed in brine with 8-12% (w/v) of salt allowing spontaneous fermentation due to autochthonous microbiota, mainly yeasts and lactic acid bacteria (LAB). Microorganism metabolic activities transform raw to fermented olives with peculiar organoleptic characteristics (Bleve et al., 2015).

In the past major interest was focused on LAB, nowadays the attention is also turned in yeast's technological and functional properties favour aiming to produce improved table olives. Focusing on positive features of yeasts, they can enhance the olives organoleptic characteristics (Arroyo-Lopéz, Querol, Bautista-Gallego, & Garrido-Fernandez, 2008), degrade polyphenols (Arroyo-Lopéz et al., 2012), act as olive antioxidant agents (Abbas, 2006), enhance the LAB growth by vitamins synthesis and inhibit non-desirable fungi (Viljoen, 2006), degrade anti-nutritional compounds (Olstorpe, Schnüren, & Passoth, 2009), fortify the olives with folates (Moslehi-Jenabian, Lindegaard Pedersen, & Jespersen, 2010), reduce the cholesterol levels (Kourelis et al., 2010), and absorb mycotoxins (Bonatsou, Tassou, Panagou, & Nychas, 2017).

Wickerhamomyces anomalus, *Candida boidinii*, *Candida diddensiae*, *Saccharomyces cerevisiae*, *Pichia guillermondii*, *Pichia membranefaciens*, *Kluyveromyces marxianus*, *Debaryomyces hansenii*, *Pichia kudriavzevii*, *Pichia fermentans*, and *Candida oleophila* were reported in table olive fermentations (Bleve et al., 2014; Martorana et al., 2015; Silva, Reto, Sol, & Xavier Malcata, 2011; Tofalo, Schirone, Perpetuini, Suzzi, & Corsetti, 2012). The species can vary according to olive cultivars, geographic origin, and method of production (Chorianopoulos, Boziaris, Stamatiou, & Nychas, 2005). Therefore, different olive cultivars possess specific yeast biota whose species may reveal and/or dominate during the fermentation according to extrinsic and intrinsic factors related to the fermentation. Brine sodium chloride and pH are reported as the main parameters controlling the fermentation (Garrido-Fernández, Fernández-Díaz, & Adams, 1997). These factors affect the presence and dominance of LAB and yeasts in fermentations (Özay & Borcakli, 1996). While more

than 10% of brine salt level favours yeasts (Leventdurur et al., 2016), reducing concentration up to 8% induces mixed fermentation by LAB and yeasts giving a product with more equilibrated characteristics (Nisiotou, Chorianopoulos, Nychas, & Panagou, 2010; Tassou, Panagou, & Katsaboxakis, 2002). Also, the presence of LAB determines a decreasing of pH guaranteeing the safety of product. The spontaneous fermentation carried out modifying these parameters can affect the growth of yeasts and it is an important source of yeast species that could be used as starter in table olive production.

Nocellara messinese is one of the main Sicilian cultivars growing also in some areas of Calabria (South Italy) and it is used to produce both oil and fermented table olives. To date, no information are reported about the microbiota composition of *Nocellara messinese* fermented olives. Poiana and Romeo (2006) enumerated LAB and yeasts of this cultivar; other authors studied the microbiota of two similar cultivars, *Nocellara del Belice* (Aponte et al., 2010) and *Nocellara etnea* (Randazzo, Todaro, Pino, Pitino, Corona, & Caggia, 2017).

In this study we studied the yeast biota associated to naturally fermented table olives of the cultivar *Nocellara messinese* profiling the evolution of the yeast consortia upon different brines. The results increase the knowledge about yeast variability connected to a cultivar and its growth environment and they could be useful for the selection of starter to be used in the fermentation process.

2. Materials and methods

2.1. Sample origin and fermentative conditions

Olives from the cultivar *Nocellara messinese*, produced by olive groves located in Calabria, were used. They were harvested at ripening period suitable for processing, immediately transported to the laboratory where they were selected, washed by water and processed by natural fermentation. The

fermentations were carried out in duplicate in screw capped plastic vats containing about 11 kg of olives and filled with about 16.5 L of brines formulated as follow: trial (a) 8% NaCl (w/v) (as control), trial (b) 8% NaCl (w/v) acidified to pH 4.3 with lactic acid, trial (c) 5% NaCl (w/v) for 20 days and then brought to 8% NaCl (w/v), trial (d) 5% NaCl (w/v) acidified to pH 4.3 with lactic acid and after 20 days brought to 8% NaCl (w/v). In each vat plastic discs were placed upon the olives to maintain them inside the brine. The fermentations were maintained at room temperature for 240 days. Salt concentration was adjusted to 8% for the trials (c) and (d) after 20 days and maintained in the range of the 8% for all the trials throughout the fermentation process by adding coarse salt in the brine. Brines were collected in depth from the vats at 0, 3, 7, 15, 30, 60, 90, 180, and 240 days; therefore, a total of 72 brines were processed for physico-chemical, microbiological, and molecular analyses.

2.2. Physico-chemical and microbiological analyses

The pH was monitored using a pH meter (Crison basic 20, Hach Lange Spain, S.L.U.). The titratable acidity was determined by titration with 0.05 N NaOH and expressed as percent of lactic acid. Salt concentration was measured periodically by digital refractometer (DBR 047 SALT) and expressed as percent of NaCl. The physico-chemical analyses were carried out in duplicate.

The brines were analysed from 0 to 240 days to study the evolution of the microbiota associated with the different brines. Therefore, each brines were serially diluted (1:10) in a solution of NaCl 0.9% (w/v), plated in triplicate onto YPD agar (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, glucose 20 g L^{-1}, and agar 20 g L^{-1}) supplemented with 100 mg/L chloramphenicol (Liofilchem Diagnostici) to enumerate yeasts and onto de Man-Rogosa-Sharpe (MRS) agar supplemented with 100 mg/L cycloheximide (Oxoid) to enumerate LAB. Yeasts and LAB were incubated under aerobic and anaerobic conditions, respectively, at 30 °C for 2 days; then, they were enumerated and an heatmap was generated using the plotly R package (Sievert et al., 2016).

2.3 Yeasts isolation, identification, and phylogenetic analysis

At 0, 3, 7, 15, 30, 60, 90, 180, and 240 days of fermentation, colonies were randomly picked up from the highest dilution plates (Pulvirenti, Solieri, Gullo, De Vero, & Giudici, 2004; Tofalo et al., 2009). The colonies were purified by streaking on YPD agar and stored at −80 °C by Microbank™ (Pro-Lab Diagnostics, Canada). Yeasts were firstly grouped by restriction fragment length polymorphism (RFLP) and then were subjected to sequencing of the D1/D2 domain of 26S rRNA gene.

In details, DNA from yeasts (231 isolates), isolated from the two replicates of each type of fermentation and grown on YPD agar at 30 °C overnight, was extracted by InstaGene Matrix (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. Amplification reactions of the 5.8S-ITS regions were performed using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Kraková et al., 2012; White, Bruns, Lee, & Taylor, 1990) with the following amplification conditions: initial denaturation at 95 °C for 2 min; 35 cycles of 30 s 95 °C for denaturing, annealing for 30 s at 55 °C, extension for 1 min at 72 °C, and a final extension step of 10 min at 72 °C. Each PCR products were digested using *Hae*III, and *Hinf*I restriction enzymes (Sigma-Aldrich, USA) according to Martorana, Giuffrè, Capocasale, Zappia, & Sidari (2018).

Since the restriction profiles of the isolates from the two replicates of each type of fermentation were comparable, the isolates from a single replicate (114 yeasts) were further considered. A representative for each restriction profile was chosen to sequence the domain D1/D2 of the 26S rRNA region using NL1 (5' GCATATCAATAAGCGGAGGAAAAG 3') and NL4 (5' GGTCCGTGTTTCAAGACGG 3') primers (Thermo Fisher Scientific, USA) according to Martorana et al. (2018). Amplified and purified products were then sequenced (Eurofins Genomics, Germany). The obtained sequences were compared with those available at the National Center for

Biotechnology Information (NCBI) using BLASTN (Altschul et al., 1997) and submitted to GenBank (https://submit.ncbi.nlm.nih.gov/subs/genbank/) for accession numbers.

Phylogenetic tree was constructed with MEGA 7 (Kumar, Stecher, & Tamura, 2016) using a Neighbor-Joining algorithm, plus the Jukes–Cantor distance estimation method with bootstrap analyses for 1000 replicates was performed.

2.4. PCR-DGGE analysis

PCR-DGGE of a portion of the 26S rRNA were carried out on brines sampled at the end of the fermentation according to Gatto & Torriani (2004). 15 μL of the amplified products were loaded onto 8% (w/v) polyacrylamide gels (acrylamide/bisacrylamide, 37.5:1) in 1× TAE buffer containing 30 to 60% urea-formamide linear denaturating gradient (100%: 7 mol/L urea and 40% (v/v) formamide) increasing in the direction of electrophoresis. The electrophoresis was carried out using the D-code apparatus (Bio-Rad Laboratories, USA) at 130 V with running temperature of 60 °C for 7 h.

DGGE bands were excised from the gel, put in distilled water and left at 4 °C overnight. Then, DNA from bands was re-amplified with the primers LIEV-f (without GC clamp) and LIEV-r using the protocol above described. Then, PCR products were purified by Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, USA), according to the manufacturer's instructions, and sequenced (Eurofins Genomics, Germany). The sequences obtained were compared as above described and submitted to GenBank for accession numbers.

2.5. Yeast technological characterisation

Twenty-five strains isolated throughout the fermentation from the different trials were tested for technological properties. The strains were grown overnight in YPD broth at 30 °C, harvested by

centrifugation (5000 rpm for 10 min), washed once in NaCl 0.9% (w/v) solution, re-suspended to OD_{600} of 1.0 in the same solution, and spotted in duplicate in different media.

The ability to hydrolyse esters was tested for 6 days at 25 °C on medium prepared with peptone (10 g L^{-1}), NaCl (5 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.1 g L^{-1}), Tween 80 (10 g L^{-1}), and agar (20 g L^{-1}) at pH 6.8 (Buzzini & Martini, 2002). The presence of esterase activity was estimated as opaque halo around the colonies. The lipase activity was tested for 6 days at 25 °C on medium containing yeast extract (10 g L^{-1}), peptone (10 g L^{-1}), NaCl (5 g L^{-1}), commercial butter (50 g L^{-1}), and agar (20 g L^{-1}) (Gardini et al., 2006). The positive activity was visible as clear halo surrounding the colonies. The pectinolytic activity was tested on plates with YNB (6.7 g L^{-1}), apple pectin (12.5 g L^{-1}), and agar (10 g L^{-1}), adjusted to pH 4.0 with 1 N HCl (Tofalo, Perpetuini, Schirone, Suzzi, & Corsetti, 2013). After 10 days at 25 °C the activity was determined by measuring the diameter of the colonies. The β-glucosidase activity was tested for 7 days at 25 °C on plates containing arbutin (5 g L^{-1}), yeast extract (10 g L^{-1}), 1% solution of ferric ammonium citrate solution, and agar (20 g L^{-1}) (Caridi, Pulvirenti, Restuccia, & Sidari, 2005). Strains positive for this activity changed the colour of the medium from colourless to brown of variable intensity. The catalase activity was determined by adding 3% (v/v) H_2O_2 on the colonies according to Bautista-Gallego et al. (2011). The presence of this activity was revealed by bubble formation.

2.6. Statistical analysis

Data were analysed by StatGraphics Centurion XVI from StatPoint using the *Least Significant Differences* of Fisher, confidence level of 95%.

3. Results

3.1. Physico-chemical and microbiological changes

Figure 1 shows pH, total titratable acidity, and salt concentration of the different fermentations. Obviously, the induced acidification influenced the pH at the start of fermentation. The acidified brines had the lowest initial pH, followed by the (c) brine adjusted for salt concentration (5.74), and by the (a) control brine (6.05). The two acidified brines showed a similar behaviour with a constant trend until 240 days. In the no acidified brines, the pH decreased throughout the process; this was more intense in the (c) brines than in the (a) brine. At the fermentation end, the brine pH stabilised at values ranged from 3.95 to 4.28 for the fermentation (c) and (b), respectively. The brines (c) and (d) determined the lowest pH values. From 0 to 180 days of fermentation, significant statistical differences among the fermentation trials were observed. The starting acidification in brines (b) and (d) correspond to lower initial values of the titratable acidity (0.05% and 0.07%, respectively) compared to (a) and (c) brines. All the brines showed a similar trend with a gradual increase until the fermentation end. Within 15 days, salt concentration decreased as result of the equilibrium between olives and brine. Significant statistical differences were observed at 3, 15, and 120 days. The addition of coarse salt in the (a) and (b) brines maintained the concentration in the range of the 7-8%; similar values are reported for the (c) and (d) brines after 20 days when the salt concentration was brought to 8%. Significant statistical differences were observed from 7 to 240 days.

The heatmap plot depicts the yeasts and LAB count throughout the fermentation (Fig. 2). For each fermentation, the yeast load increased gradually reaching at 30 days the maximum values (range 6.61-6.97 Log UFC mL^{-1} for brines (d) and (a), respectively). Then, the yeast populations start to decrease reaching, at the end of the fermentation, values in the range of 5.33-5.92 Log UFC mL^{-1} for brines (b) and (c), respectively (Fig. 2a). Throughout the process significant statistical differences among the four fermentations were observed with yeast load values distributed in different homogeneous groups (a homogeneous group defined as a group of means within which there are no statistically significant differences). In the control brine (a) the LAB population increased gradually reaching at 30 days the maximum value (6.29 Log UFC mL^{-1}); then the LAB

concentration declines. The acidified brines (b) and (d) showed similar trend with higher LAB loads for the brine (d). In the brine (c), with 5% of salt then adjusted to 8%, the LAB increased from the third day reaching the maximum value at 60 days (6.95 Log UFC mL^{-1}); then LAB declines reaching the value of 5.73 Log UFC mL^{-1} at the fermentation end (Fig. 2b). Throughout the process significant statistical differences among the four fermentations were observed; the LAB loads were distributed in 2 and 4 homogeneous groups at the start of fermentations and in all the other sampling points, respectively.

3.2. Yeasts identification and heterogeneity

In Table S1 the ITS size, the RFLP patterns, the identification by sequencing, the percentage of similarity, and the accession numbers of the closest relative by BLAST of representative strains are reported.

Sequencing the D1/D2 of 26S of the rRNA allowed to identify the isolated yeasts. To confirm the position of each strain in phylogeny, type strains 26S sequences were retrieved from the GenBank database, and they were subjected to phylogenetic analysis together with the isolated strains sequences (Fig. 3). Based on these sequence data, a phylogenetic tree was drawn by the Neighbor-Joining method. According to Kurtzman & Robnett (1998), yeast strains that show nucleotide substitution greater than 1 % in the D1/D2 domain of the 26S rRNA are likely to represent different species. As shown in Figure 3, the phylogenetic tree of D1/D2 26S rRNA sequences confirmed the previous identification of the isolates.

P. kudriavzevii was the most isolated specie (34% of the total isolates) followed by *W. anomalus* (27%), *Z. hellenicus* (9%), *C. boidinii* (7%), *S. cerevisiae* (7%), *C. aaseri* (6%), *C. tropicalis* (4%), *C. diddensiae* (3%), *Z. meyerae* (2%), *P. mexicana* (1%).

Figure 4 reports the yeast species frequency of the different trials at each stage of the fermentation. Species of *W. anomalus* and *P. kudriavzevii* were present in all the brine formulations

264 at various stages of fermentation. The adjustment of salt concentration from 5% to 8% induced a
265 constant presence of *P. kudriavzevii* throughout the fermentation (Fig. 4c). At the start and in the
266 middle stage of fermentation, *Z. hellenicus* was found (Fig. 4d and c) while the brines with 8% of
267 salt (a) also acidified (b) delayed its presence at the last stage of fermentation (Fig. 4a and b). Also,
268 the brines (a) and (b) favoured the growth of *Candida* spp. at various stage of fermentation while
269 the adjustment of the salt concentration (c) and the low salt concentration coupled with the
270 acidification (d) delayed its presence at the last stage of fermentation. Comparing the acidified
271 brines (Fig. 4b and d), at the middle stages of fermentation the adjusted for salt concentration and
272 acidified brine (d) induced the dominance of *S. cerevisiae* while the other acidified brine (b) was
273 characterised by the presence of *C. diddensiae* and *C. boidinii*. At the end of fermentation, *W.*
274 *anomalus* was found in all the brines. Moreover, other species were *Z. hellenicus* in (a) and (b), *P.*
275 *kudriavzevii* in (a) and (c), and *C. aaseri* in (c) and (d). *P. mexicana* and *S. cerevisiae* species were
276 found only in the acidified brine with 8% of salt (b) and in the acidified brine with salt
277 concentration adjusted from 5% to 8% (d), respectively.
278
279 *3.3. PCR-DGGE*
280
281 For all the brines at the end of fermentation, *W. anomalus* was identified by comparison with *W.*
282 *anomalus* strain isolated from plates and reference strains. This identification was confirmed by
283 sequencing considering a similarity of 99%. The accession numbers of the bands sequenced and
284 deposited to GenBank are: band from brine (a) MH485382, band from brine (b) MH485383, band
285 from brine (c) MH485384, band from brine (d) MH485385.
286
287 *3.4. Yeast technological properties*
288

289 Table 1 reports the biochemical activities of the twenty-five strains tested. The esterase and lipase
290 activities ranged from absence to strong activities. The strains positive for esterase activity were
291 16% and belonged to the species *C. tropicalis*, *C. diddensiae*, and *Z. meyerae* while for lipase
292 activity were 24% and belonged to the species *C. tropicalis*, *C. diddensiae*, *C. boidinii*, *C. aaseri*, *P.*
293 *mexicana*, *W. anomalus*, and *Z. meyerae*. All the strains grown on the medium supplied with pectin.
294 The 64% of the strains exhibited from light to strong β-glucosidase activity and they belonged to
295 the species *C. tropicalis*, *C. diddensiae*, *C. aaseri*, *P. mexicana*, *S. cerevisiae*, *W. anomalus*, *Z.*
296 *meyerae*, and *Z. hellenicus*. The 52% of the strains had catalase activity and they belonged to the
297 species *C. tropicalis*, *C. boidinii*, *P. kudriavzevii*, *S. cerevisiae*, and *W. anomalus*.
298
299 **4. Discussion**
300
301 This study assessed the yeast ecology changes of green table olives fermented using brine
302 formulations different for salt concentration and pH. All the tested brine formulations led to pH
303 values that guarantee the safety of the product.
304 A broad range of yeast biodiversity in the fermentation of *Nocellara messinese* olives was
305 revealed. In this study, 5 genera are detected and 10 species: *P. kudriavzevii*, *W. anomalus*, *C.*
306 *boidinii*, *Z. hellenicus*, *S. cerevisiae*, *C. aaseri*, *C. tropicalis*, *C. diddensiae*, *Z. meyerae*, and *P.*
307 *mexicana*. The PCR-DGGE highlighted lower yeast biodiversity compared to the diversity found by
308 culture-dependent method. Anyway, other authors have reported similar discrepancies (Di Maro,
309 Ercolini, & Coppola, 2007). At the end of the fermentations the specie *W. anomalus*, revealed by
310 culture-independent approach, was also isolated and identified by culture-dependent method.
311 The shift of salt concentration from 5% to 8% delayed the growth of some species at the end of
312 the fermentation. The acidification with lactic acid determined a growth of the species *P. mexicana*
313 and *S. cerevisiae* not found in the other brine formulations. The use of different brine formulations
314 allowed us both to study the change of the yeast ecology of green olive of the cultivar *Nocellara*

315 *messinese* and to isolate strains of different species. Knowing the yeast actors during the
316 fermentation process and those becoming dominants at its end has importance from the
317 technological point of view; especially taking into consideration the yeast strain properties that can
318 be correlated to the product final organoleptic characteristics.

319 Potential benefit of yeast starters in table olive production is related mainly to the aromatic
320 profile improvement due to yeast esterase and lipase activities, the olives preservation from
321 oxidation due to yeasts positive for catalase, the olive debittering due to β-glucosidase activity
322 (Anagnostopoulos, Bozoudi, & Tsaltas, 2017). This latter property has technological importance for
323 the reduction of NaOH used in the olive debittering process. Yeast starter should have no or low
324 pectinolytic activity to not determine olive spoilage; on the other hand, various authors reported that
325 pectinolytic yeasts are common in olive fermentations and give a positive contribution to texture
326 and flavour of table olives (Arroyo-Lopéz et al., 2008; Golomb et al., 2013).

327 Concerning the technological characterisation for starter selection, it is interesting to highlight
328 the biodiversity of the species here identified in terms of presence of the different enzymatic
329 activities and also the strain dependent distribution of the activities inside a species.

330 The strains lipase and esterase activities enhances the olive organoleptic characteristics
331 (Bonatsou et al., 2017). We observed the lipase activity in *C. boidinii* and *Zygoascus* spp. as
332 reported by other authors (Rodríguez-Gómez, Arroyo-López, López-López, Bautista-Gallego, &
333 Garrido-Fernández, 2010) but also in other species of *Candida* and in *P. mexicana*. Concerning the
334 esterase activity, Hernández, Martin, Aranda, Pérez-Nevado, & Córdoba (2007) reported different
335 species of *Candida* and strains of *S. cerevisiae* possessing this enzymatic activity. Our results
336 showed the lowest number of strains belonging to the genera *Candida* and *Zygoascus* having the
337 esterase activity compared to the other activities tested. Variability in pectin degradation was
338 reported (Bevilacqua, Beneduce, Sinigaglia, & Corbo, 2013; Hernández et al., 2007). Our results
339 are in agreement with the data reported by Hernández et al. (2007) for the colony size. Anyway, the
340 presence of these yeasts did not determine softening spoilage. β-glucosidase has an important role

in fermented olives both for secondary metabolites production and for the hydrolysis of oleuropein as well (Arroyo-Lopéz et al., 2012; Anagnostopoulos, Bozoudi, & Tsaltas, 2017). Different species of *Candida* and *Pichia* (Lilao et al., 2015; Tofalo et al., 2013), *W. anomalus* (Restuccia et al., 2011), and *S. cerevisiae* (Hernández, Espinosa, Fernández-González, & Briones, 2003; Sirilun et al., 2016) were reported to possess β-glucosidase activity. We detected the strongest activity in *W. anomalus*, *Z. meyerae*, *Z. hellenicus*, and *C. diddensiae*. Species with lower activity were *P. mexicana*, *C. aaseri*, *C. tropicalis*, and *S. cerevisiae*. Strains of *C. tropicalis* and *C. boidinii*, *P. kudriavzevii*, *S. cerevisiae*, and *W. anomalus* exhibited catalase activity suggesting that they could contribute to the protection of green table olives from fatty acid oxidation and peroxide formation. Our findings are in agreement with findings of other authors (Silva et al., 2011; Tofalo et al., 2013).

The species which were here isolated and tested have been reported to possess other interesting properties: enhancing the growth of LAB (Segovia Bravo et al., 2007), killer properties towards non-desired fungi (Hernández et al., 2008; Llorente et al., 1997), degrading anti-nutritional compounds (Moslehi-Jenabian et al., 2010; Olstorpe et al., 2009), reducing cholesterol (Kourelis et al., 2010), and mycotoxins detoxification (Shetty and Jespersen, 2006).

Some of the yeasts here isolated and studied could be good candidates as starter for table olive fermentation: *C. diddensiae* L945 (medium lipase activity, low pectinolytic activity, and very high β-glucosidase activity), *C. aaseri* L1182 (medium lipase activity, low pectinolytic activity, and very high β-glucosidase activity), *P. mexicana* L1155 (high β-glucosidase activity and low pectinolytic activity), *W. anomalus* L1231 (high β-glucosidase activity, low pectinolytic activity, and medium catalase activity), *Z. meyerae* L1172 (medium lipase and esterase activities, low pectinolytic activity, and very high β-glucosidase activity). Other strains such as *C. diddensiae* L937 and those belonging to *W. anomalus* specie possess very high β-glucosidase activity; therefore, they could be used in mixture with other strains possessing the other interesting enzymatic activities.

Our results may be useful to optimise the industrial-scale green olive fermentations in order to improve the debittering process and the quality of the fermented olives. Also, this study is a starting

point with the future perspectives of screening the isolated strains of the different species towards other peculiar features for the production either of improved table olives or other fermented food preparations possessing functional characteristics. Future perspective could be the genetic improvement of the spore-forming strains by micromanipulation, in order to obtain descendants improved for the positive searched characters but lacking other characters negative for the food production, and the evaluation of the effective bioavailability of substances such as vitamins and antioxidant compounds produced by yeasts ascertaining, working together with different kinds of expertise, the real health benefits.

Conflict of interest

The authors declared no conflict of interest.

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Figure captions

Fig. 1. pH, titratable acidity and salt concentration changes in the brines of *Nocellara messinese* table olives naturally fermented with different brine formulations. (a) 8% NaCl (w/v); (b) 8% NaCl (w/v) acidified with lactic acid to pH 4.30; (c) 5% NaCl (w/v) for 20 days and then brought to 8% NaCl (w/v); (d) 5% NaCl (w/v), acidified using lactic acid to pH 4.30 and after 20 days brought to 8% NaCl (w/v). Data are mean values of duplicate fermentations ± standard deviation.

Fig. 2. Heatmaps summarising yeast a) and LAB b) counts in the different fermentation trials.

Fig. 3. Phylogenetic tree based on 26S rRNA gene D1/D2 domain sequences of the isolates. The tree was constructed by the Neighbor-Joining method and the Jukes-Cantor model. Numerals represent the confidence level from 1000 replicate bootstrap samplings (frequencies less than 55% are not indicated). Bar indicates the distance corresponding to one base change per hundred nucleotide positions.

Fig. 4. The frequency, expressed as percentage, of yeast species (■ *C. aaseri*, ■ *C. boidinii*, ■ *C. diddensiae*, ■ *C. tropicalis*, ■ *P. kudriavzevii*, ■ *P. mexicana*, ■ *S. cerevisiae*, ■ *Z. meyerae*, ■ *W. anomalus*, ■ *Z. hellenicus*) at each stage of the different fermentations; a trial (a) 8% NaCl (w/v), b trial (b) 8% NaCl acidified to pH 4.3 with lactic acid, c trial (c) 5% NaCl for 20 days and then brought to 8% NaCl, and d trial (d) 5% NaCl acidified to pH 4.3 with lactic acid and after 20 days brought to 8% NaCl.

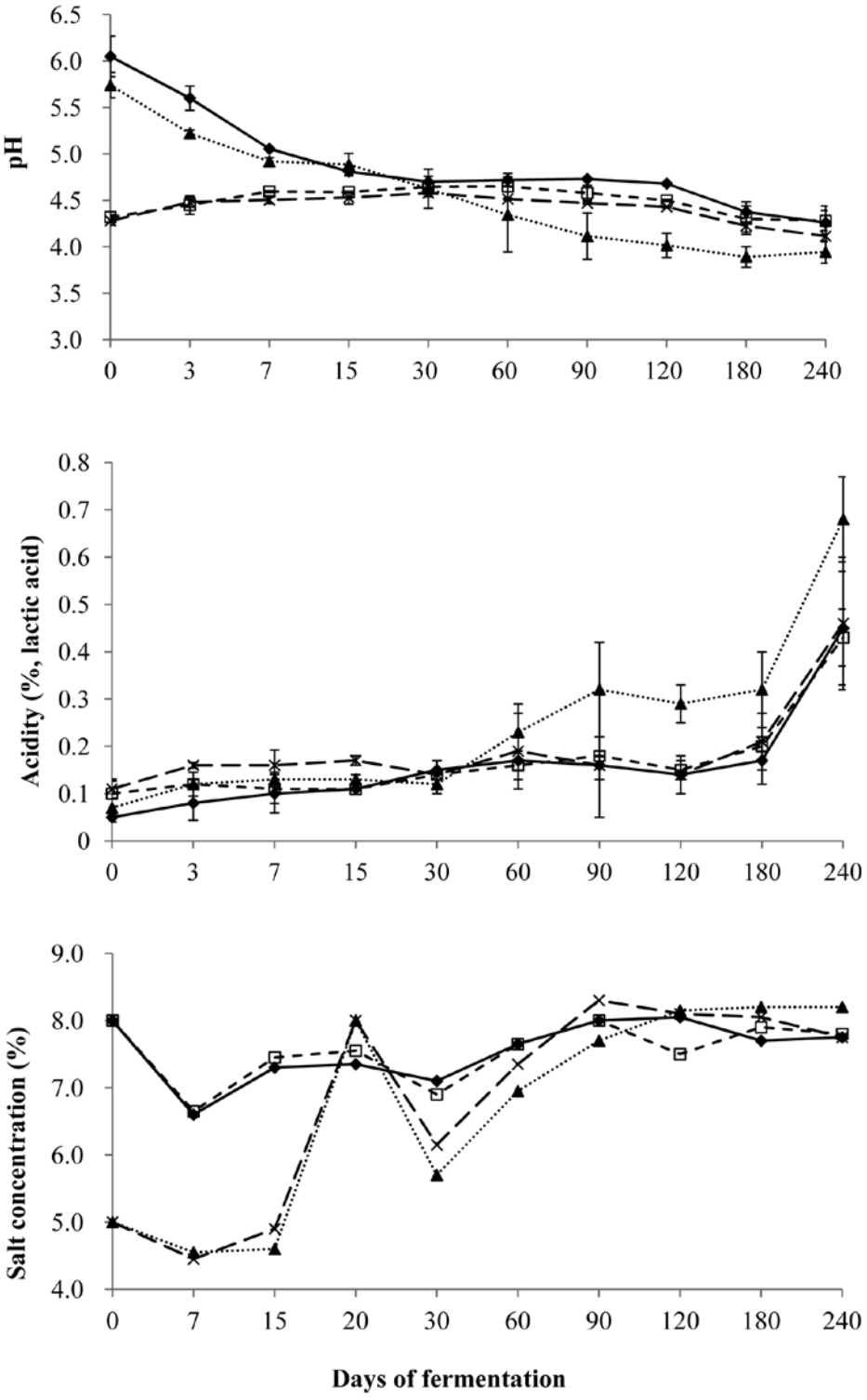
pH
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
0
3
7
15
30
60
90
120
180
240
Acidity (%, lactic acid)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Salt concentration (%)
9.0
8.0
7.0
6.0
5.0
4.0
20
Days of fermentation

Fig. 1

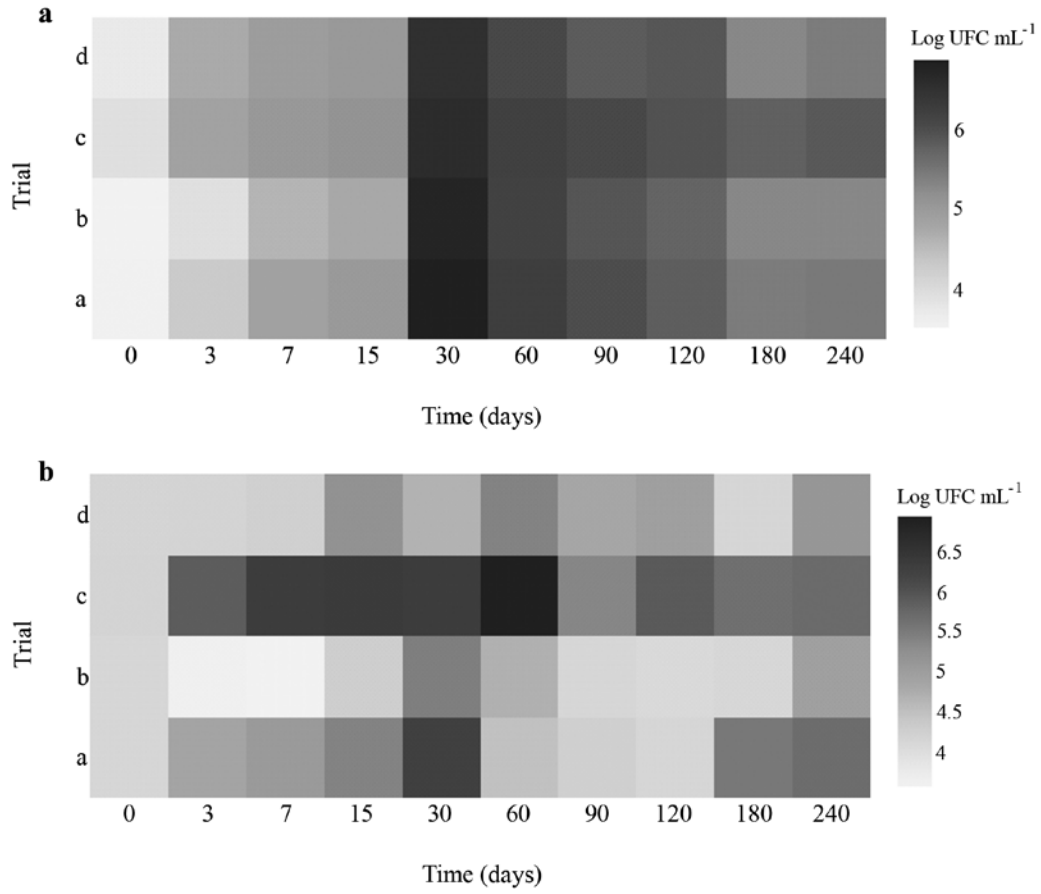
a
Trial
d
c
b
a
0
3
7
15
30
60
90
120
180
240
Time (days)
Log UFC mL-1
6
5
4
b
Trial
d
c
b
a
0
3
7
15
30
60
90
120
180
240
Time (days)
Log UFC mL-1
6.5
6
5.5
5
4.5
4

Fig. 2

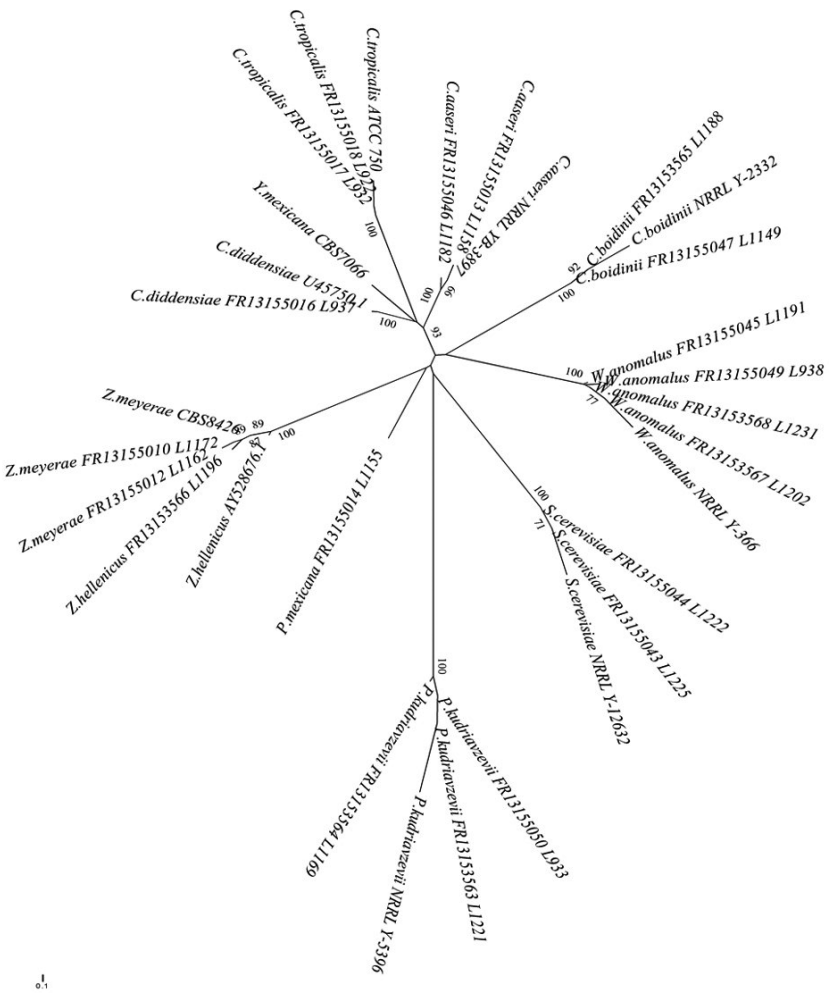
C.tropicalis ATCC 750
C.tropicalis FR13155018 L922
C.tropicalis FR13155017 L932
C.auseri FR13155046 L1182
C.auseri FR13153013 L1158
C.auseri NRRL YB-3897
C.boidinii FR13153565 L1188
C.boidinii NRRL Y-2332
C.boidinii FR13155047 L1149
Y.mexicana CBS7066
C.diddensiae U45750.1
C.diddensiae FR13155016 L931
W.anomalus FR13155045 L1191
W.anomalus FR13155049 L938
W.anomalus FR13153568 L1231
W.anomalus FR13153567 L1202
W.anomalus NRRL Y-366
Z.meyerae CBS8426
Z.meyerae FR13155010 L1172
Z.meyerae FR13155012 L1162
Z.hellenicus FR13153566 L1196
Z.hellenicus AY528676.1
P.mexicana FR13155014 L1155
S.cerevisiae FR13155044 L1222
S.cerevisiae FR13155043 L1225
S.cerevisiae NRRL Y-12632
P.kudriavzevii FR13155050 L933
P.kudriavzevii FR13153563 L1221
P.kudriavzevii NRRL Y-5396
P.kudriavzevii FR13153564 L1169

Fig. 3

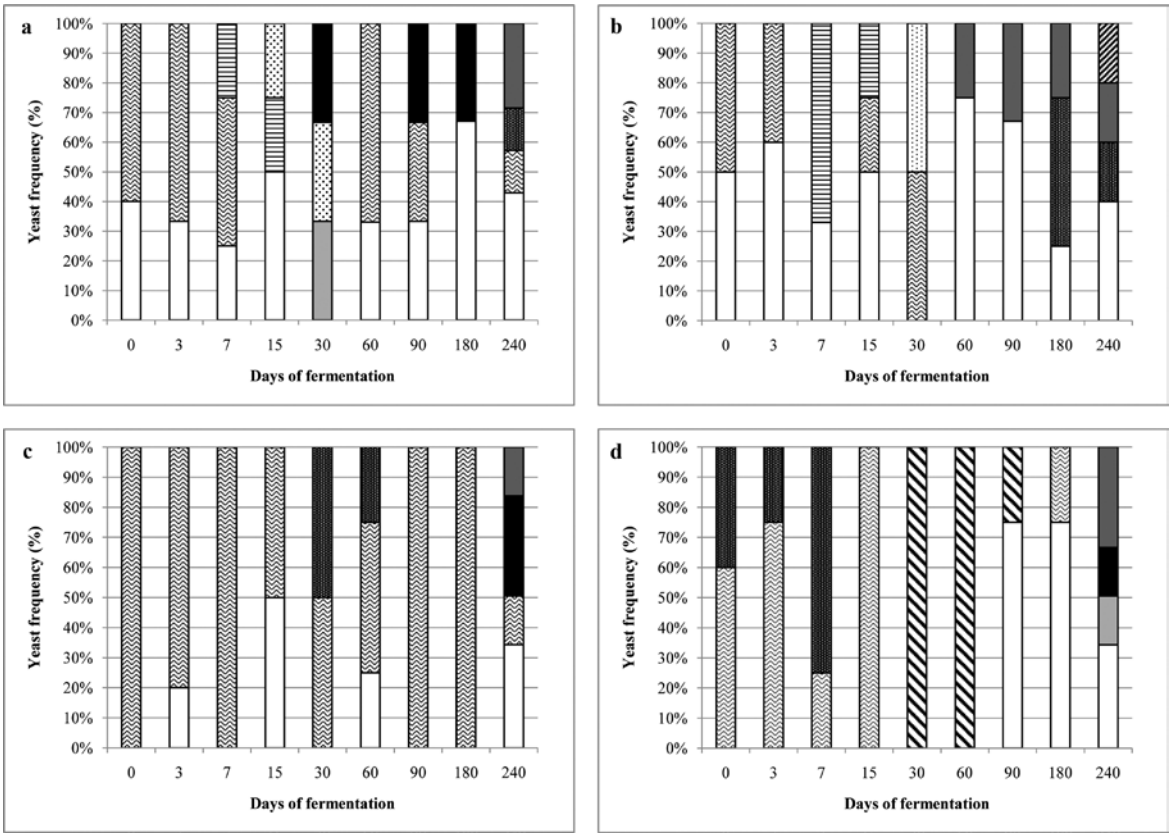
a
b
c
d
Yeast frequency (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
3
7
15
30
60
90
180
240
Days of fermentation

Fig. 4

611 **Table 1** Technological properties of yeast strains isolated from the different olive brines.

Yeast strains	**Technological properties**				
	Esterase activity[a]	Lipase activity[b]	Pectinolytic activity[c]	β-glucosidase activity[d]	Catalase activity[e]
C. tropicalis L922	-	-	+	+	+
C. tropicalis L932	+	+	+	-	+
C. diddensiae L937	-	-	++	++++	-
C. diddensiae L945	+	++	+	++	-
C. boidinii L1149	-	-	+	-	+
C. boidinii L1165	-	+	+	-	-
C. boidinii L1188	-	-	+	-	-
C. aaseri L1158	-	++	++	++	-
C. aaseri L1182	-	++	+	++	-
P. kudriavzevii L933	-	-	++	-	+++
P. kudriavzevii L946	-	-	+	-	+++
P. kudriavzevii L1169	-	-	+	-	+
P. kudriavzevii L1221	-	-	+	-	+++
P. mexicana L1155	-	+	+	+++	-
S. cerevisiae L1222	-	-	+	-	+
S. cerevisiae L1225	-	-	+	+	-
W. anomalus L938	-	-	++	++++	+
W. anomalus L1179	-	-	++	++++	++
W. anomalus L1191	-	+	++	++++	+
W. anomalus L1231	-	-	+	+++	++
W. anomalus L1202	-	-	++	++++	+++
Z. meyerae L1162	++	+	+	++++	-
Z. meyerae L1172	++	++	+	++++	-
Z. hellenicus L1196	-	-	+	+++	-
Z. hellenicus L1197	-	-	+	++++	-

[a]Activity: -, no opaque halo; +, faint opaque halo; ++, strong opaque halo.

[b]Activity: -, no clear halo; +, faint clear halo; ++, strong clear halo.

[c]Diameter of the colonies: +, 2-5 mm; ++, > 5 mm.

[d]Activity: -, no brown colour; +, very light brown; ++, light brown; +++, brown; ++++, dark brown.

[e]Activity: - , no activity; +, very weak activity; ++, weak activity; +++, strong activity.

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Table S1 PCR-RFLP analysis of the 5.8-ITS rRNA and percentage of similarity by Blast of yeast species isolated from different fermentations.

Strains	Species	ITS (bp)	*Hae*III	*Hinf*I	% Similarity (accession no. of the closest relative by Blast)
L938	*Wickerhamomyces anomalus*	680	620	310	99% (HM107788)
L1191	*Wickerhamomyces anomalus*	600	600	110+120+350	99% (EF532302)
L1231	*Wickerhamomyces anomalus*	620	600	150+210	100% (MH237950)
L1202	*Wickerhamomyces anomalus*	600	600	280+310	99% (MF574472)
L933	*Pichia kudriavzevii*	500	90+400	155+250	99% (LC177025)
L1221	*Pichia kudriavzevii*	500	380	300	99% (EU394711)
L1169	*Pichia kudriavzevii*	500	390	150+210	99% (JF715183)
L1155	*Pichia mexicana*	610	150+450	340	99% (EU807898)
L937	*Candida diddensiae*	650	70+130+450	320	99% (KY106416)
L1149	*Candida boidinii*	700	700	150+180+350	99% (KY106342)
L1188	*Candida boidinii*	700	700	110+120+350	100% (KY296061)
L932	*Candida tropicalis*	530	480	260	100% (MH260384)
L922	*Candida tropicalis*	500	80+450	250	99% (JN185908)
L1158	*Candida aaseri*	610	130+440	150+160+300	99% (KY106269)
L1182	*Candida aaseri*	700	80+150+400	160+180+300	100% (KY106269)
L1222	*Saccharomyces cerevisiae*	850	130+160+230+300	150+210	99% (HM101471)
L1225	*Saccharomyces cerevisiae*	850	130+160+230+300	300	99% (HM191663)
L1162	*Zygoascus meyerae*	610	600	100+160+330	100% (KY110228)
L1172	*Zygoascus meyerae*	700	600	120+180+350	100% (KX610375)
L1196	*Zygoascus hellenicus*	600	600	300	100% (NG_055323)