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Evaluation of physiological properties of yeast strains isolated from olive oil and their *in vitro* probiotic trait

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ABSTRACT

Virgin olive oil contains a biotic fraction represented by rich microbiota, including yeasts. The aim of this study was to investigate some physiological properties and the *in vitro* probiotic potential of yeast strains previously isolated from Italian virgin olive oil. Eleven yeast strains belonging to the species *Candida adriatica*, *Candida diddensiae*, *Nakazawaea molendini-olei*, *Nakazawaea wickerhamii*, *Wickerhamomyces anomalus*, and *Yamadazyma terventina* were used in this study and compared with the reference yeast *Saccharomyces boulardii*. Present research has demonstrated that unlike *Saccharomyces boulardii* which produce only satured and monounsatured fatty acids (MUFAs), the olive oil-borne yeast strains also synthesize polyunsatured fatty acids (PUFAs) in quantities greater than those found in olive oil, which provide health benefits. The survival in gastric and pancreatic juices, which is important for probiotic yeasts because it allows them to cross the human intestinal tract, has reached a maximum of 100% when yeast cells were coated with olive oil. Cholesterol was removed by 50% of the studied yeast strains, and among them, the best results were reached by the strains 2032 and 2033 of *W. anomalus* which appear the best probiotic candidate in terms of the *in vitro* probiotic trait evaluated. Further experiments are underway to confirm this findings.

1. Introduction

Virgin olive oil is a basic component of the Mediterranean diet and is well-known throughout the world for its sensorial and nutritional value. It is generally accepted that a virgin olive oil-rich diet provides many health benefits, such as the reduction of cardiovascular diseases and certain types of cancers and modifications of inflammatory responses (Justino et al., 2012). The nutritional and health benefits have been ascertained considering the chemical components of olive oil, such as tocopherols, carotenoids and polar phenolic compounds (Petroni et al., 1995; Visioli et al., 1995). Despite the importance of the abiotic component of olive oil, recent research has demonstrated that freshly produced virgin olive oil contains a biotic fraction represented by a rich microbiota, including yeasts (Ciafardini and Zullo, 2002). The presence of these yeasts in the newly produced virgin olive oil is mainly due to their migration from the carposphere of the olives to the olive oil during the extraction process (Ciafardini et al., 2017). Some yeasts of the carposphere of the olives do not survive for a long time in the oily habitat, whereas others reproduce in a selective way, according to the chemical composition of the product, and become the typical microbiota of the olive oil (Ciafardini et al., 2004; Zullo et al., 2010). The presence and activity of yeasts in virgin olive oil were only discovered in the last two decades, so the studies carried out on the microbiology of olive oil are relatively few and focused mainly on some biotechnological aspects (Ciafardini et al., 2006 a,b; Romo-Sánchez et al., 2010; Zullo and Ciafardini, 2008; Zullo et al., 2010). On the other hand, little is known about whether olive oil-borne yeasts, in addition to the functions discovered so far, have potential beneficial effects in human health as probiotics. Probiotics are defined as live microorganisms that have beneficial effect in the health of the host when ingested (FAO/WHO, 2001). Probiotics have established their efficacy when consumed as food components or dietary supplements. Probiotic strains must be harmless, non-toxic to the host, survive the gastrointestinal transit, whereas beneficial effects to the host include cholesterol reduction, production of vitamins, antibacterial and antioxidant activity, as well as enhancement of the immune system (McFarland and Bernasconi, 1993). Despite the multiple attributes of a large number of yeast strains isolated from foods, only two species have been recognized as probiotics: *Saccharomyces cerevisiae* and *Saccharomyces boulardii* which is a member of the *S. cerevisiae* species. The supplementation of *S. cerevisiae* live cultures in animals has been reported to improve growth, health and immune response in the hosts (Shen et al., 2009). *S. boulardii,* isolated from the litchi fruit in Indochina by Henri Boulard in the 1920s, is used to treat diarrhea in adults and children infected with *Clostridium*

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difficile, diarrhea in human immunodeficiency virus-infected patients, and acute and chronic diarrhea in children and adults (Czerucka et al., 2007; McFarland and Bernasconi, 1993; Ouwehand et al., 2002). Other yeast species, such as *Debaryomyces hansenii*, *Torulaspora delbrueckii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Kluyveromyces lodderae*, *Candida norvegica*, and *Galactomyces relssii*, have shown tolerance to passing through the gastrointestinal tract and an ability to inhibit enteropathogens (Kourelis et al., 2010; Oliveira et al., 2017; Psani and Kotzekidou, 2006). Nowadays it is not known whether the biotic component of virgin olive oil, represented by yeasts, can implement the already known health benefits of the product due to its chemical components. The living yeast cells of virgin olive oil normally are ingested by the consumers, since the regulation establishes that this food, after being produced, can not be manipulated with additional chemical and physical treatments, including pasteurization. However the behavior and role that the oil-borne yeasts play in the gastro-intestinal tract remains unknown. Considering the lack of knowledge described above, the aim of the present research was to investigate *in vitro* physiological and probiotic properties of yeast strains belonging six species previously isolated from mono varietal virgin olive oil of Leccino and Taggiasca cultivars.

2. Materials and methods

2.1. Yeast strains, culture media and fatty acid methyl esters (FAMEs)

Yeast strains belonging to the species *Candida adriatica*, *Candida diddensiae*, *Nakazawaea molendini-olei*, *Nakazawaea wickerhamii*, *Wickerhamomyces anomalus*, and *Yamadazyma terventina* were studied in the present research as probable probiotic sources. All of the yeast strains were previously isolated with high frequency from Italian virgin olive oil produced by the Leccino and Taggiasca cultivars (Ćadeź et al., 2012; Ciafardini et al., 2013b, 2017; Ciafardini and Zullo, 2015, 2018; Zullo et al., 2010). The yeast strains belonging to each species were chosen for their best biotechnological properties in the starter selection. The identification was accomplished by sequencing the approximately 600 base-pair D1/D2 region of the large (26S) ribosomal subunit using primers NL1 and NL4, as described by Kurtzman and Robnett (1997). A commercial lyophilized *Saccharomyces boulardii* Codex (Zambon Italia, s.r.l. –Bresso-Milan) was used as a probiotic reference yeast strain. All yeast cultures, stored at −40 °C in a sterile glycerol solution (20%, v/v), were grown on MYGP agar medium containing 3 g of yeast extract, 3 g of malt extract, 2.5 g of soy peptone, 2.5 g of bacto tryptone, 10 g of D-glucose and 1000 mL distilled water with a pH of 7 and incubated at 30 °C for 5 days (Kurtzman and Fell, 1998). The oil-borne yeast strains and the reference *S. boulardii* were characterized on the basis of the chemical composition of the yeast lipids. Fatty acid composition was analyzed according to the Commission Regulation (EC) No. 796/2002 (EC, 2002) and the amending Regulation EC 2568/91 of the European Commission (EC, 2013), with some modifications. The cultures were grown on MYGP agar at 30 °C for 48 h, and 0.3 g of wet cells were harvested through centrifugation (6000 × *g* for 5 min) and transferred respectively into 10 mL glass screw-cap tubes. The yeast fatty acids were saponified by adding 1 mL of a 1.125 M NaOH aqueous methanol solution (1:1, v/v) and heating at 100 °C in a water bath (Argo Lab, mod. Wb-12, Italy) for 30 min. The FAMEs derivatives were prepared by adding 2 mL of a 6 N aqueous methanol HCl solution (46:54, v/v) and heating at 80 °C in a water bath for 10 min. FAMEs were extracted from the aqueous phase with 1.25 mL of a hexane-methyl tert-butyl mixture (1:1, v/v), and 3 mL of a diluted solution of 0.3 M NaOH in water was used to remove residual reagents from the organic extracts. The upper solvent phase was transferred into 2 mL glass vials and evaporated for 12 h at room temperature. The dry extract of the FAMEs was stored at −20 °C until analysis. The dry extract was solubilized using heptane and individual FAMEs from the yeast cells were separated and quantified using gas chromatography equipment (Perkin-Elmer) equipped with a flame ionization detector (FID) and an SP-2560 capillary column (100 m × 0.25 μm film x 0.20 d.i.) from SUPELCO (USA). Hydrogen was used as a carrier gas with a flow rate of 25.0 mL/min. The oven was set to the following temperature program: 60 °C (the initial temperature) for 3 min, increased to 170 °C at a rate of 5 °C/min for 9 min, then to 230 °C at a rate of 10 °C/min for 5 min. The injector and detector temperatures were set at 250 °C and 270 °C, respectively, and the injection volume was 1 μL. Fatty acids peak identification was accomplished by comparing the peak retention times with standard compounds from Sigma (St. Louis, MO, USA) injected under the same gas chromatographic condition. The results were expressed as relative percent of total area. Three replicates were prepared and analyzed per sample.

2.2. Hydrophobicity

The hydrophobicity of the yeast strains was assessed according to the method of Rosenberg (2006) with few modification. The yeast cultures grown for 24 h at 30 °C in the MYGP broth were harvested by centrifugation at 6000 × *g* for 5 min (Universal 32 centrifuge, Hettich, Tuttlinger, Germany) and washed twice with PBS (0.1 M, pH 7.4). The cell count was adjusted to approximately 10^9 colony forming unit (CFU) per mL. Two milliters of the cell suspension of each yeast strain was mixed with an equal volume of *n*-hexadecane by vortexing for 2 min. The aqueous and organic phases were allowed to separate by maintaining the mixture in an undisturbed state for 1 h. Next, the aqueous layer was gently removed with a pipette and the OD_{600} was measured. The cell surface hydrophobicity was calculated using the following equation: hydrophobicity (%) = ($ABS_{initial}$-ABS_{final}/$ABS_{initial}$) x 100, where $ABS_{initial}$ represents the initial absorbance before mixing and ABS_{final} represents the final absorbance after mixing with *n*-hexadecane. The experiment with three replications was repeated twice.

2.3. Biofilm formation

The biofilm formation was evaluated using a sterile polyethylene 24-wells plate containing 2 mL of sterile MYGP broth. After inoculation with 0.5 mL of each yeast culture with a number of CFU equal to about Log 8.77 and the same volumes of sterile distilled water as a control, the plates were incubated overnight at 30 °C. After incubation, the liquid was discarded, and the biofilm-coated wells of the plates were washed twice with mL sodium phosphate buffer 0.5 M pH 7 and air dried at room temperature for 45 min. Then, the wells were stained with a 0.4% aqueous crystal violet solution for 45 min. Subsequently, each well was washed four times with sterile distilled water and destained with 2 mL of 95% ethanol. After 45 min of incubation, 1.5 mL of destaining solution from each well was analyzed at 595 nm with a spectrophotometer, comparing the results with the control. The experiment with three replications was repeated twice.

2.4. Decarboxylation activity

The ability of the yeast strains to decarboxylate amino acids, producing biogenic amines, was assessed as described by Gardini et al. (2006). Aliquots of 0.1 g/L of glucose, 0.06 g/L of bromocresol purple (Sigma-Aldrich), 1 g/L of each amino acid (L-tyrosine, L-histidine, L-arginine), and 15 g/L of agar were dissolved in 900 mL of distilled water. After sterilization, 100 mL of Yeast Nitrogen Base (Difco) solution (6.7%, w/v), previously sterilized by filtration (Minisart NML – Sartorius), was aseptically added, and the final pH was adjusted to 5.3 using HCl. Differences between all of the yeast strains were observed in the plates containing all of the amino acids. After 4 days of incubation at 25 °C, a positive or negative designation, based on the presence or absence of a violet halo surrounding the colonies, was given to each of the assayed yeast strains and recorded.

2.5. Autoaggregation capacity

The autoaggregation percentage of the yeast strains was determined using the method described by Gil-Rodriguez et al. (2015), with a few modifications. A sample (4 mL) of the yeast culture grown in MYGP broth for 24 h at 30 °C was harvested by centrifugation (5000 × *g* for 5 min, 4 °C), washed twice with a sterile solution of 0.9% NaCl (w/v), re-suspended in the same volume of a 0.5 M pH 7.4 sodium phosphate buffer solution and stored in disposable plastic at 37 °C. At the beginning (A_0) and after 4 h (A_t) of incubation, 100 μL was removed from the upper surface without shaking, mixed with 900 μL of a 0.5 M pH 7.4 phosphate buffer solution and analyzed with a spectrophotometer at an OD equal to 600 nm. The autoaggregation percentage was calculated as follows: Autoaggregation (%) = $(1\text{-}A_t/A_0)$ x 100.

The experiment, with three replications, was repeated twice.

2.6. In vitro simulated gastric and pancreatic digestion with olive oil-free yeast cells

In vitro gastric (GD) and pancreatic digestions (PD) were performed using the methodology described by Bonatsou et al. (2015). For the GD step, a synthetic gastric juice was prepared in a buffer solution at pH 2.0 containing NaCl (2.05 g/L), KH_2PO_4 (0.60 g/L), $CaCl_2$ (0.11 g/L) and KCl (0.37 g/L). The pH was adjusted with 1 M HCl. After the sterilization of the medium, pepsin (0.0133 g/L) and lysozyme (0.01 g/L) from Sigma-Aldrich (St. Louis, USA) were added to complete the gastric solution. The assayed yeast cultures were allowed to grow in the MYGP broth (24 h at 30 °C) and were then separated by centrifugation at 6000 × *g* per 10 min. The pellet was washed twice with a sterile 0.9% (w/v) NaCl solution. The cells of each yeast strain were then re-suspended in 30 mL of the gastric solution, adjusting the cell concentration to about 10^6 per mL by Direct Microscopic Count (DMC) utilizing a Thoma cell counting chamber. The tubes with the yeast strains suspended in the 0.9% (w/v) NaCl solution (control) were used for the microbiological analysis at the beginning of the incubation period, while those with the gastric solution were incubated for 2.5 h at 37 °C in an orbital shaker set at 100 rpm to simulate the peristaltic movements. The PD simulation was carried out using a buffer solution with pH of 8.0, adjusted with 1 M HCl, formulated with bile salts (3.0 g/L), pancreatin (0.1 g/L), Na_2HPO_4 (26.9 g/L) and NaCl (8.5 g/L). Harvested cells from the previous GD step were centrifuged (5000 × *g* for 5 min), washed twice with the sterile 0.9% (w/v) NaCl solution and re-suspended in 30 mL of the same solution. Then, 5 mL were used for the microbiological analysis while 25 mL were centrifuged as before, and the pellet was re-suspended in 25 mL of the simulated pancreatic solution. After 3.5 h of incubation at 37 °C with agitation using a thermostatic orbital shaker (100 rpm), the yeast cultures suspended in the simulated pancreatic solution were centrifuged at 5000 × *g* for 10 min. Then, the pellets, were washed twice as before and re-suspended in the same volume of sterile 0.9% (w/v) NaCl. The plating on the MYGP agar medium at the beginning and at the end of each digestive simulation was performed to assess cell viability. For each yeast strain, three independent repetitions were performed.

2.7. In vitro simulated gastric and pancreatic digestion tests with olive oil-coated yeast strains

The *in vitro* simulated gastric and pancreatic digestion test with yeast cells coated with olive oil was performed in three independent assays according to the Bonatsou et al. (2015) method, with a few modifications. For the GD and PD simulation, the synthetic gastric and pancreatic juices were prepared as described above. The assayed yeast cultures were grown in MYGP broth for 24 h at 30 °C and were then collected by centrifugation at 6000 × *g* for 10 min. The pellets were washed twice with a sterile 0.9% (w/v) NaCl solution and re-suspended in sterile 0.9% (w/v) NaCl. For each yeast strain, the cell concentration was corrected with the DMC to 10^6 per mL. The solutions were divided into three plastic test tubes, each with 30 mL of solution, and centrifuged again at 6000 × *g* (4 °C) for 10 min. The pellet of each test tube was suspended in 1 mL of filter-sterilized virgin olive oil (Minisart NML-Sartorius, Göttingen, Germany) through 10 min of vortex agitation. Then, 29 mL of sterile synthetic gastric juice and 29 mL of sterile synthetic pancreatic juice were added to each test tube and vortex mixed. The yeast cultures were incubated in agitation for 3.5 h at 37 °C. After incubation, the yeast cells were collected by centrifugation (6000 × *g* for 10 min), washed twice, re-suspended in the same volume of sterile 0.9% (w/v) NaCl solution, and analyzed as described before. The plating on the MYGP agar medium at the beginning (zero time) and at the end of each digestive simulation were performed to assess cell viability. The virgin olive oil used for the yeast cell suspensions was extracted from the Italian cv. Leccino, and the chemical composition was close to that of the initial olive oil samples from which some of the studied oil-borne yeast strains were isolated.

2.8. Cholesterol assimilation

A freshly prepared Yeast Nitrogen Base (Difco) with L-histidine was used as a basal medium and was supplemented with pure water-soluble cholesterol (Sigma-Aldrich) and ox gall bile salt (Sigma-Aldrich) as follows: YNB, Yeast Nitrogen Base (0.67 g/L) and L-histidine (0.01 g/L); YNB + CG: YNB, cholesterol (0.182 g/L) and glucose (10 g/L); YNB + CGO: YNB + CG, and ox gall (3 g/L); and YNB + CO: YNB, cholesterol, and ox gall. All media were filter sterilized and transferred aseptically into sterile empty test tubes with screw caps. The yeast strains grown in the MYGP broth overnight at 30 °C were removed by centrifugation at 5000 × *g* for 5 min then suspended in a sterile 0.9% (w/v) NaCl solution in which the cell concentration was adjusted with the DMC to 10^7 per mL. After 2 h of starvation at 30 °C, the yeast strains were inoculated at a 1% level in the media described above and incubated for 48 h at 37 °C. After the incubation period, cells were removed by centrifugation (9000 × *g* for 15 min), and the remaining cholesterol in the spent broth was determined colorimetrically at an OD equal to 600 nm, using the reagents and procedure included in the Cholesterol Quantitation Kit (MAK043) provided by Sigma-Aldrich (St. Louis, MO, USA). All experiments were replicated twice.

2.9. Statistical analysis

A priori one-way analysis of variance, using Tukey's HSD (honest significant difference) test, was performed using the Statgraphics computer program (Statgraphics, version 6, Manugistics, Inc. Rockville, MA). Statistical significance was indicated at $p < 0.05$. A cluster analysis of similarity indices was performed with version 13.2.1 of the JMP statistical software from SAS Institute (USA).

3. Results and discussion

3.1. Physiological properties

3.1.1. Fatty acid profiles of the yeasts

Fatty acids are a source of cellular energy and serve as building blocks for a number of complex membrane lipids. The gas-chromatographic analysis of the fatty acid methyl esters (FAMEs) in yeasts consists of several steps, such as the esterification of lipids, sample injection, separation, identification and quantification (Eder, 1995). Acid-catalyzed transesterification is the most common method used for the preparation of FAMEs, which is used in various applications (Glaser et al., 2010). FAMEs analysis using the gas-liquid chromatography method has successfully been applied in the identification of clinically important yeast species (Kutty and Philip, 2008). Kurtzman and Fell (1998) reported that long chain fatty acids of C16:0 and C18:0 are predominant in yeasts, including palmitic (16:0), palmitoleic (C16:1),

Table 1
Average fatty acids composition of olive oil-born yeast strains in hundred percent.

Free fatty acid	Olive oil	Sbo	Yt 1987	Yt 1988	Cd 1918	Cd 1922	Ca 1985	Ca 2036	Nm 1926	Nm 2035	Nw 1885	Wa 2032	Wa 2033
Myristic acid	0.01^{d}	0.89^{ab}	0.68^{ab}	0.12^{c}	0.82^{ab}	1.76^{a}	0.45^{b}	1.59^{a}	0.44^{b}	0.18^{c}	0.22^{bc}	0.29^{bc}	0.38^{b}
Palmitic acid	16.03^{ab}	16.15^{ab}	21.75^{a}	10.93^{b}	22.06^{a}	19.38^{a}	14.68^{ab}	14.98^{ab}	14.39^{ab}	12.31^{ab}	9.41^{b}	16.23^{ab}	20.53^{a}
Palmitoleic acid	1.07^{d}	47.01^{a}	6.93^{bc}	9.17^{b}	7.92^{bc}	7.71^{bc}	7.91^{bc}	7.21^{bc}	9.17^{b}	9.09^{b}	9.52^{b}	4.80^{c}	4.99^{c}
Heptadecanoic acid	0.04^{d}	0.00	0.44^{b}	0.54^{ab}	0.00	0.00	0.39^{b}	0.32^{b}	0.69^{a}	0.60^{a}	0.65^{a}	0.24^{c}	0.34^{b}
Heptadecenoic acid	0.07^{d}	0.66^{c}	1.00^{bc}	3.95^{a}	0.00	1.95^{b}	1.52^{b}	3.90^{a}	1.79^{b}	3.19^{a}	4.05^{a}	0.93^{bc}	1.05^{b}
Stearic acid	1.77^{cd}	5.82^{a}	2.46^{c}	2.21^{c}	1.86^{cd}	1.58^{cd}	3.42^{a}	1.97^{c}	1.95^{c}	0.88^{d}	0.98^{d}	1.87^{cd}	3.05^{b}
Oleic acid	69.80^{a}	26.49^{c}	42.06^{b}	33.26^{c}	30.00^{c}	23.83^{c}	39.81^{b}	33.63^{c}	32.51^{c}	31.01^{c}	32.96^{c}	32.42^{c}	38.51^{bc}
Linoleic acid	9.79^{d}	0.00	14.85^{c}	36.96^{a}	24.38^{b}	30.00^{ab}	23.97^{b}	27.05^{ab}	28.50^{ab}	33.50^{a}	37.31^{a}	35.56^{a}	26.36^{b}
Arachic acid	0.32^{c}	0.00	0.59^{c}	0.00	0.00	6.68^{a}	0.18^{d}	0.00	0.46^{c}	6.02^{a}	2.05^{b}	3.67^{b}	0.00
Linolenic acid	0.62^{e}	0.00	8.32^{ab}	1.81^{d}	12.75^{a}	6.20^{b}	6.81^{b}	3.35^{bc}	8.94^{ab}	2.99^{c}	2.21^{c}	3.40^{c}	3.97^{bc}
Eicosenoic acid	0.27	0.00	0.06	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00
Behenic acid	0.10	0.00	0.19	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.13	0.15	0.00
Lignoceric tR acid	0.04^{d}	2.76^{a}	0.36^{c}	0.35^{c}	0.00	0.62^{b}	0.22^{c}	5.44^{a}	0.48^{bc}	0.06^{d}	0.00	0.03^{d}	0.38^{c}
Total %	99.93	99.78	99.69	99.30	99.79	99.71	99.61	99.56	99.32	99.83	99.49	99.59	99.56
SFA	18.31	25.62	26.47	14.15	24.74	30.02	19.34	24.42	18.41	20.05	13.44	22.48	24.68
MUFA	71.21	74.16	50.05	46.38	37.92	33.49	49.49	44.74	43.47	43.29	46.53	38.15	44.55
PUFA	10.41	0.00	23.17	38.77	37.13	36.20	30.78	30.40	37.44	36.49	39.52	38.96	30.33

Different letter in the same line indicate significant differences by Tukey's test ($P < 0.05$). Olive oil (reference lipid), Sbo: *Saccharomyces boulardii* (reference yeast), Yt 1987: *Yamadazyma terventina* 1987, Yt 1988: *Yamadazyma terventina* 1988, Cd 1918: *Candida diddensiae* 1918, Cd 1922: *Candida diddensiae* 1922, Ca 1985: *Candida adriatica* 1985, Ca 2036: *Candida adriatica* 2036, Nm 1926: *Nakazawaea molendini-olei* 1926, Nm 2035: *Nakazawaea molendini-olei* 2035, Nw 1885: *Nakazawaea wickerhamii* 1885, Wa 2032: *Wickerhamomyces anomalus* 2032, Wa 2033: *Wickerhamomyces anomalus* 2033. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids. The average fatty acid composition of the olive oil-borne yeast strains and the reference *S. boulardii* yeast are illustrated in Table 1. Results revealed the existence of thirteen fatty acids in the yeast strains isolated from virgin olive oil and seven in *S. boulardii*. The most abundant fatty acids in the *S. boulardii* yeast cells were palmitoleic acid (47.01%) and oleic acid (26.49%), followed by palmitic acid (16.15%) and stearic acid (5.82%), with minor amounts of lignoceric tR acid (2.76%), myristic acid (0.89%) and heptadecenoic acid (0.66%). In contrast, the long-chain fatty acids, such as linoleic acid (C18:2), arachic acid (C20:0), linolenic acid (C18:3), eicosenoic acid (C20:1) and behenic acid (C22:0), were not detected and seemed to be absent (Table 1). These results were in agreement with the results obtained by other studies carried out on *S. cerevisiae* by Avery et al. (1996) and Bassam et al. (2014), who noticed that the percentages of palmitoleic fatty acid (C16:1) were 55.7% and 50.24%, respectively, and with Yazawa et al. (2009) which reported the absence of polyunsatured fatty acids (PUFAs). The mean relative percentage of fatty acids found in the cellular material of the olive oil-borne yeasts varied according to the strains of yeasts studied. This behavior has also been demonstrated in other studies carried out on other yeast species (Kock and Botha, 1998). The analysis of the hierarchical tree showed different clusters where *S. boulardii* tended to be the most dissimilar compared to the other yeast strains. The FAMEs profile of *C. diddensiae* strains 1918 and 1922 were similar to *W. anomalus* 2033 and *Y. terventina* 1988, and these samples formed one cluster that was different from *S. boulardii* (Fig. 1). The fatty acid profile of olive oil, which is the original habitat from which the yeast strains studied had been isolated, is different compared to that of the yeasts. Overall, compared to olive oil, all yeasts, including *S. boulardii*, have been shown to be richer in saturated fatty acids (SFAs). In respect to monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids, the yeast strains isolated from the oleic ecosystem showed a different behavior compared to that of the reference yeast. In fact, the yeast strains from olive oil had a percentage of MUFAs, which was from 21.16% (*Y. terventina* 1987) to 37.72% (*C diddensiae* 1922) lower than that of the olive oil, while the percentage of PUFAs was from 12.76% (*Y. terventina* 1987) to 29.11% (*N. wickerhamii* 1885) higher than that of olive oil (Table 2). Linoleic acid (C18: 2) and linolenic acid (C18: 3) are two PUFAs considered to be essential fatty acids, which, together with oleic acid (C18: 1), are involved in reducing the risk of coronary heart

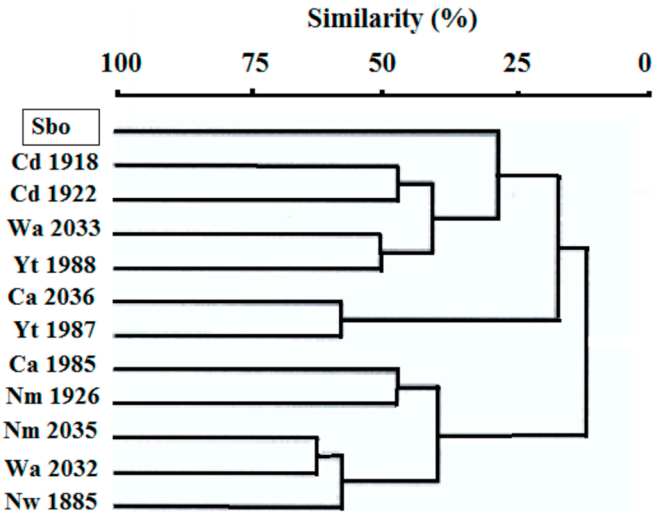

Fig. 1. Dendrogram from the cluster analysis of the fatty acid profile of the yeast strains. (Sbo: *Saccharomyces boulardii* (reference yeast), Ca 1985: *Candida adriatica* 1985, Ca 2036: *Candida adriatica* 2036, Cd 1918: *Candida diddensiae* 1918, Cd 1922: *Candida diddensiae* 1922, Nm 1926: *Nakazawaea molendini-olei* 1926, Nm 2035: *Nakazawaea molendini-olei* 2035, Nw 1885: *Nakazawaea wickerhamii* 1885, Wa 2032: *Wickerhamomyces anomalus* 2032, Wa 2033: *Wickerhamomyces anomalus* 2033, Yt 1987: *Yamadazyma terventina* 1987, Yt 1988: *Yamadazyma terventina* 1988).

disease. In virgin olive oil, within the MUFAs group, oleic acid is predominant (69.80%), which differentiates it from other vegetable oils. Virgin olive oil is poor in PUFAs, in fact, within the PUFAs group, linoleic and linolenic acids were found at very low concentrations (Table 1). From the results reported in Table 2, it is possible to observe that, contrary to the reference yeast, *S. boulardii*, all oil-borne yeast strains are richer in linoleic acid (C18: 2) by a minimum of 5.06% (*Y. terventina* 1987) to a maximum of 27.52% (*N. wickerhamii* 1885) and richer in linolenic acid (C18: 3) by between 1.19% (*Y. terventina* 1988) and 12.13% (*C. diddensiae* 1918). The high content of PUFAs in olive oil-borne yeast strains could be important, because the numerous yeast cells suspended in the freshly produced oil, following their autolysis,

Table 2
Difference between percentage of the yeasts and the virgin olive oil FAMEs.

Free fatty acid	Sbo	Yt 1987	Yt 1988	Cd 1918	Cd 1922	Ca 1985	Ca 2036	Nm 1926	Nm 2035	Nw 1885	Wa 2032	Wa 2033
Myristic acid (C14:0)	0.88	0.67	0.11	0.81	1.75	0.44	1.58	0.43	0.17	0.21	0.28	0.37
Palmitic acid (C16:0)	0.12	5.72	−5.10	6.03	3.35	−1.35	−1.05	−1.64	−3.72	−6.62	0.20	4.50
Palmitoleic acid (C16:1)	45.94	5.86	8.10	6.85	6.64	6.84	6.14	8.10	8.02	8.45	3.73	3.92
Heptadecanoic acid (C17:0)	−0.04	0.40	0.50	−0.04	−0.04	0.35	0.28	0.65	0.56	0.61	0.20	0.30
Heptadecenoic acid (C17:1)	0.59	0.93	3.88	−0.07	1.88	1.45	3.83	1.72	3.12	3.98	0.86	0.98
Stearic acid (C18:0)	4.05	0.69	0.44	0.09	−0.19	1.65	0.20	0.18	−0.89	−0.79	0.10	1.28
Oleic acid (C18:1)	−43.31	−27.74	−36.54	−39.80	−45.97	−29.99	−36.17	−37.29	−38.79	−36.84	−37.38	−31.29
Linoleic acid (C18:2)	−9.79	5.06	27.17	14.59	20.21	14.18	17.26	18.71	23.71	27.52	25.77	16.57
Arachic acid (C20:0)	−0.32	0.27	−0.32	−0.32	6.36	−0.14	−0.32	0.14	5.70	1.73	3.35	−0.32
Linolenic acid (C18:3)	−0.62	7.70	1.19	12.13	5.58	6.19	2.73	8.32	2.37	1.59	2.78	3.35
Eicosenoic acid (C20:1)	−0.27	−0.21	−0.27	−0.27	−0.27	−0.02	−0.27	−0.27	−0.27	−0.27	−0.27	−0.27
Behenic acid (C22:0)	−0.10	0.09	−0.10	−0.10	−0.10	−0.10	0.02	−0.10	−0.10	0.03	0.05	−0.10
Lignoceric tR acid (C24:0)	2.72	0.32	0.31	−0.04	0.58	0.18	5.40	0.44	0.02	−0.04	−0.01	0.34
SFA	7.31	8.16	4.16	6.43	11.71	1.03	6.11	0.10	1.74	4.87	4.17	6.37
MUFA	2.95	−21.16	−24.83	−33.29	−37.72	−21.72	−26.47	−27.74	−27.92	−24.68	−33.06	−26.66
PUFA	−10.41	12.76	28.36	26.72	26.37	20.37	19.99	27.03	26.08	29.11	28.55	19.92

Sbo: *Saccharomyces boulardii* (reference yeast), Yt 1987: *Yamadazyma terventina* 1987, Yt 1988: *Yamadazyma terventina* 1988, Cd 1918: *Candida diddensiae* 1918, Cd 1922: *Candida diddensiae* 1922, Ca 1985: *Candida adriatica* 1985, Ca 2036: *Candida adriatica* 2036, Nm 1926: *Nakazawaea molendini-olei* 1926, Nm 2035: *Nakazawaea molendini-olei* 2035, Nw 1885: *Nakazawaea wickerhamii* 1885, Wa 2032: *Wickerhamomyces anomalus* 2032, Wa 2033: *Wickerhamomyces anomalus* 2033. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

can free the PUFAs with the possibility of improving the acidic composition of the olive oil during its storage, or in the kitchen during their cooking, which may have health benefits in the long run. The cultivation of such olive oil-borne yeasts could be useful for the production of single cell oils (SCO) rich in PUFAs to be used to balance the acidic composition of vegetable oils.

3.1.2. Hydrophobicity

The hydrophobicity of each yeast strain was measured using cell suspensions in a phosphate buffer solution and hexadecane as a hydrophobic phase. A total of 72% of the olive oil-borne yeast species studied, as well as the reference *S. boulardii*, showed significant differences related to their hydrophobicity (Table 5). Both *C. adriatica* 1985 (55.50%) and *Y. terventina* 1987 (45.50%) demonstrated the highest level of hydrophobicity, while hydrophobicity was negative in *N. molendini-olei* strains 1926 and 2035 and *N. wickerhamii* 1885. According to Wenzhou et al. (2011), yeast cell hydrophobicity is an important factor determining yeast enrichment in the oily phase during olive oil extraction in the mill.

3.1.3. Biofilm formation

The ability of the olive oil-borne yeast strains to form biofilms on abiotic surfaces was evaluated *in vitro* using a plastic surface. In total, 73% of the studied yeast strains demonstrated the ability to adhere to the plastic surface and form a biofilm. *Y. terventina* strains 1987 and 1988 and *W. anomalus* 2033 formed the best biofilms, while *C. adriatica* 2036 and the strains 1926 and 2035 of *N. molendini-olei* did not differ significantly compared to the reference *S. boulardii* (Table 5). The high percentage of oil-borne yeasts able to adhere to the plastic surface can be explained due to their dimorphism. Zullo et al. (2010) reported that the oleic system contains a high number of dimorphic yeasts, which produce pseudohyphae for nutrients under starving conditions. The decreased biofilm-forming ability demonstrated by the two oil-borne strains of *N. molendini-olei* confirm the findings of Porru et al. (2018) obtained from *N. molendini-olei* strains isolated from the brines of table olives. Considering that in some low-quality olive oils, it is possible to find some opportunistic pathogen yeasts, such as *Candida parapsilosis* (Zullo et al., 2010), *Candida guilliermondii* and *Cryptococcus carnescens* (Santona et al., 2018), the ability of dimorphic yeasts to form biofilms *in vitro* is not always positive for human health because in pathogenic yeasts, it is considered as an index of pathogenicity (Kalai Chelvam et al., 2014). However, good-quality virgin olive oils that are rich in phenolic compounds do not allow for the survival of unwanted yeasts for long periods during product storage (Ciafardini et al., 2013a). Other types of oils can be microbiologically improved through filtration.

3.1.4. Decarboxilase activity

The decarboxylase activity of the assayed yeasts, assessed in a mix of L-tyrosine, L-histidine and L-arginine, is presented in Table 5. Yeast cultures grown in the presence of amino acids showed a surrounding halo and were recorded as positive. In the present study, the decarboxylase activity was yeast species-dependent, and only the strains 2032 and 2033 of *W. anomalus* were decarboxylase positive. However, other authors demonstrated similar results for other species of yeasts isolate from olive oil (Santona et al., 2018). The decarboxylation of amino acids represents a potential negative activity because it leads to the formation of biogenic amines in fermented foods and beverages in which several precursor amino acids are present (Torrea Goni and Aucìn Azpilicueta, 2001). In the case of virgin olive oil, although specific studies are lacking, it is possible to hypothesize that decarboxylation does not represent a health risk as it is prevented by the lack of amino acids and by the inhibitory activity of polar phenolic compounds present in the oleic ecosystem.

3.2. Probiotic potential

3.2.1. Autoaggregation capacity

The autoaggregation capacity is reflected by the ability of microorganisms to join together forming cellular aggregates, thereby favoring gastro-intestinal colonization (García-Cayuela et al., 2014). The autoaggregation capacity of the assayed yeasts is reported in Table 5. After 4 h of incubation at 37 °C, the yeast strains exhibited high variability in autoaggregation percentages, ranging between 8.33% (*N. molendini-olei* 2035) and 47.50% (*C. diddensiae* 1922). *C. diddensiae* 1922 and *S. boulardii* showed autoaggregation percentages that were significantly higher ($P < 0.05$) than those in the other yeasts. The variability in these results indicates that the autoaggregation ability is strongly dependent on the yeast species and strains, as suggested by Gil-Rodríguez et al. (2015). This ability is an important prerequisite for the colonization of probiotic strains in the gastro-intestinal tract because it prevents their immediate elimination by the peristaltic movements and allows for a competitive advantage among autochthonous microorganisms (Syal and Vohra, 2013).

Table 3
Yeast strains survival to *in vitro* simulated gastric and pancreatic digestion.

Yeast strains	Survival of olive oil-uncoated yeast strains (%)			Survival of yeast strains coated with olive oil (%)			
	Gastric digestion	Pancreatic digestion	Overall digestion	Gastric digestion	Δ*	Pancreatic digestion	Δ
Sbo	66.06 (7.10)^{a}	6.92 (1.54)^{a}	4.57 (0.95)^{a}	71.89 (8.50)^{ab}	5.83	35.84 (5.12)^{bc}	28.92
Yt 1987	25.70 (4.72)^{cd}	2.45 (1.05)^{b}	0.63 (0.15)^{c}	93.91 (12.20)^{a}	68.21	100.05 (10.50)^{a}	97.06
Yt 1988	10.12 (1.25)^{de}	1.41 (0.52)^{bc}	0.14 (0.05)^{d}	60.81 (9.18)^{b}	50.69	99.90 (15.60)^{a}	98.49
Cd 1918	3.01 (0.88)^{e}	0.28 (0.10)^{d}	< 0.01 (0.00)^{e}	35.13 (5.30)^{c}	32.12	27.90 (5.12)^{c}	24.89
Cd 1922	1.78 (0.38)^{e}	0.62 (0.12)^{c}	0.01 (0.00)^{e}	13.60 (1.12)^{d}	11.82	99.50 (15.20)^{a}	98.88
Ca 1985	30.20 (3.80)^{c}	6.92 (0.92)^{a}	3.19 (0.02)^{a}	33.09 (5.23)^{c}	2.89	18.61 (2.35)^{d}	11.69
Ca 2036	16.60 (3.40)^{d}	7.59 (1.52)^{a}	3.01 (0.07)^{a}	17.06 (0.90)^{d}	0.46	17.84 (2.40)^{d}	10.25
Nm 1926	3.80 (1.26)^{e}	0.25 (0.05)^{d}	< 0.01 (0.00)^{e}	< 0.01 (0.00)^{e}	−3.80	11.22 (5.20)^{d}	10.97
Nm 2035	38.41 (4.92)^{bc}	0.31 (0.05)^{d}	0.12 (0.05)^{d}	< 0.01 (0.00)^{e}	−38.41	4.57 (1.12)^{e}	4.26
Nw 1885	75.86 (5.12)^{a}	2.34 (0.80)^{b}	1.78 (0.07)^{b}	78.62 (10.30)^{ab}	2.76	16.72 (2.52)^{d}	14.38
Wa 2032	17.78 (2.30)^{d}	0.08 (0.00)^{e}	< 0.01 (0.00)^{e}	100.88 (13.22)^{a}	83.10	47.86 (3.85)^{b}	47.78
Wa 2033	50.12 (3.81)^{b}	0.35 (0.07)^{d}	0.17 (0.05)^{d}	55.64 (8.20)^{b}	5.52	27.89 (2.82)^{c}	27.54

*Δ, Difference between olive oil-coated and uncoated yeast strains survival. Data are reported as mean and standard deviation (in parenthesis) obtained from triplicate trials. Different letter in the same column indicate significant differences by Tukey's test ($P < 0.05$). Sbo: *Saccharomyces boulardii* (reference yeast), Yt 1987: *Yamadazyma terventina* 1987, Yt 1988: *Yamadazyma terventina* 1988, Cd 1918: *Candida diddensiae* 1918, Cd 1922: *Candida diddensiae* 1922, Ca 1985: *Candida adriatica* 1985, Ca 2036: *Candida adriatica* 2036, Nm 1926: *Nakazawaea molendini-olei* 1926, Nm 2035: *Nakazawaea molendini-olei* 2035, Nw 1885: *Nakazawaea wickerhamii* 1885, Wa 2032: *Wickerhamomyces anomalus* 2032, Wa 2033: *Wickerhamomyces anomalus* 2033.

3.2.2. *In vitro* simulated gastric and pancreatic digestion with olive oil-free yeast cells

Resistance to gastric and pancreatic juices is a very important feature of probiotic yeast strains, as it allows them to cross the human digestive tract. So far, some studies have focused specifically on the probiotic potential of yeasts isolated from table olives (Bonatsou et al., 2015; Silva et al., 2011), while studies on oil-borne yeasts are almost non-existent. Santona et al. (2018), using cultures of yeasts suspended in aqueous solutions, reported that, among 64 yeasts isolated from Sardinian olive oil, 40 were resistant to a pH level of 2.5 and 55 were resistant to 1.5% bile salt. However, it is important to note that, unlike other habitats where yeasts often live in watery matrices, in the olive oil ecosystem, yeasts are suspended in oily matrices surrounding the cells. In this case, yeasts have a good chance of changing their responses to external environment, which in this case is represented by gastric and pancreatic juices. For this purpose, the ability of oil-borne yeast strains to survive in simulated gastric and pancreatic conditions was investigated using the same yeast cultures suspended directly in aqueous matrices and yeast cultures pre-coated with virgin olive oil before their introduction into aqueous matrices. The results from the *in vitro* digestion test, conducted with uncoated and olive oil-coated yeast cells, are reported in Table 3. Tests performed with strains of yeasts not coated with olive oil showed survival rates between 1.78% and 75.86%, depending on the yeast strain studied. The *S. boulardii* (66.06%), used as a probiotic yeast reference, and *N. wickerhamii* 1885 (75.86%) showed the highest survival rates during *in vitro* gastric digestion, while the other yeast species (*Y. terventina*, *C. diddensiae*, *C. adriatica*, *N. molendini-olei*, and *W. anomalus*) showed significantly lower survival percentages, with the exception of *C. diddensiae*. In general, the survival rates of the same yeast strains after simulated *in vitro* pancreatic digestion were lower compared with gastric digestion (Table 3). In this case, *S. boulardii* (reference strain) and *C. adriatica* strains 1985 and 2036 demonstrated the highest survival rates after pancreatic digestion of 6.92%, 6.92% and 7.59%, respectively. The survival of the other yeast strains varied from a minimum of 0.25% (*N. molendini-olei* 1926) to a maximum of 2.45% (*Y. terventina* 1987). The lower survival rates of *S. boulardii* in the simulated *in vitro* pancreatic digestion and the olive oil-borne yeast strains in respect to gastric digestion (Table 3) are in accordance with the results of other research on yeast strains isolated from table olives (Oliveira et al., 2017; Porru et al., 2018). Regarding the overall survival of the simulation of both digestive process (gastric and pancreatic processes), it was observed that *S. boulardii* showed the greatest to the entire digestive process with a value of 4.57%. This value was statistically similar to that of *C. adriatica* strains 1985 and 2036, while *N. molendini-olei* 1926, *C. diddensiae* strains 1918 and 1922, and *W. anomalus* 2032 showed the lowest resistance (Table 3).

3.2.3. *In vitro* simulated gastric and pancreatic digestion with olive oil-coated yeast strains

On the other hand, tests performed with the same yeast strains coated with virgin olive oil have shown interesting success in the *in vitro* simulated gastric and pancreatic digestion processes. In general, resistance to *in vitro* gastric digestion, with the exception of two yeast strains *N. molendini-olei* 1926 and 2035, increased in all of the olive oil-coated yeasts compared with the uncoated yeasts. The olive oil-coated yeast strains *W. anomalus* 2032 (100.88%), *Y. terventina* 1987 (93.91%), *N. wickerhamii* 1885 (78.62%), and *S. boulardii* (71.89%) showed the highest survival rates after *in vitro* gastric digestion, which, compared to the uncoated yeast strains, increased by 83.10%, 68.21%, 2.76%, and 5.83%, respectively (Table 3). It is interesting to note that, with the exception of the two strains *N. molendini-olei* 1926 and 2036, all the other coated yeasts, including *S. boulardii* used as a reference, benefitted from the protective action of olive oil during the *in vitro* simulation of gastric digestion. This behavior can be explained by considering the hydrophobic activity of the olive oil layer present on the cell surface, which impeded the direct contact of the yeast cells with the gastric juice. The positive results regarding the survival of the yeasts was even more evident when the olive oil-coated yeast strains were subjected the *in vitro* simulation of pancreatic digestion. In this case, the survival rates varied from a minimum of 4.57% (*N. molendini-olei* 2035) to a maximum of 100.05% (*Y. terventina* 1987). Compared to the results of the same uncoated yeast strains, the survival rates were increased by a minimum of 4.26% (*N. molendini-olei* 2035) to a maximum of 98.88% (*C. diddensiae* 1922), depending on the yeast strain studied (Table 3). Compared to *in vitro* gastric digestion, the olive oil-coated yeast strains were expected to be less protected during the *in vitro* simulation of pancreatic digestion, since the bile salts of the pancreatic juice are active on lipids. It is possible to hypothesize that the short time of incubation (3.5 h) was not sufficient to completely remove the olive oil layer surrounding the yeast cells. This is the first time it has been reported that the suspension of yeast cells in virgin olive oil, with a medium polar phenol content, enhances the yeast survival in the presence of gastric and pancreatic juices. This aspect is interesting under a practical point of view because it allows for a new way of taking *S. boulardii* that is available in the market.

Table 4
Cholesterol removal (%) of oil-borne yeasts in YNB medium with cholesterol + glucose (CG), YNB with cholesterol + glucose + ox gall (CGO), and YNB with cholesterol + ox gall (CO).

Yeast strains	CG	CGO	CO
Sbo	35.38 (5.32)^{bc}	18.42 (2.43)^{c}	0.12 (0.07)^{c}
Yt 1987	8.39 (0.84)^{d}	0.99 (0.06)^{e}	0.00
Yt 1988	34.50 (6.45)^{bc}	23.91 (2.20)^{c}	0.00
Cd 1918	6.29 (0.62)^{d}	0.80 (0.05)^{e}	0.00
Cd 1922	22.11 (4.32)^{c}	9.98 (1.99)^{d}	0.00
Ca 1985	3.15 (0.32)^{d}	0.99 (0.01)^{e}	0.00
Ca 2036	26.77 (4.23)^{c}	46.44 (0.41)^{b}	20.67 (2.21)^{b}
Nm 1926	47.66 (4.65)^{b}	43.00 (2.65)^{b}	13.28 (1.34)^{bc}
Nm 2035	41.24 (6.43)^{b}	31.68 (4.12)^{bc}	7.23 (0.80)^{c}
Nw 1885	39.60 (4.20)^{b}	30.50 (4.20)^{bc}	8.40 (0.60)^{c}
Wa 2032	62.73 (6.13)^{a}	63.71 (4.19)^{a}	36.28 (5.21)^{a}
Wa 2033	58.57 (6.22)^{a}	61.93 (0.35)^{a}	42.11 (6.10)^{a}

Data are reported as mean and standard deviation (in parenthesis) obtained from triplicate trials. Different letter in the same column indicate significant differences by Tukey's test ($P < 0.05$). Sbo: *Saccharomyces boulardii* (reference yeast), Yt 1987: *Yamadazyma terventina* 1987, Yt 1988: *Yamadazyma terventina* 1988, Cd 1918: *Candida diddensiae* 1918, Cd 1922: *Candida diddensiae* 1922, Ca 1985: *Candida adriatica* 1985, Ca 2036: *Candida adriatica* 2036, Nm 1926: *Nakazawaea molendini-olei* 1926, Nm 2035: *Nakazawaea molendini-olei* 2035, Nw 1885: *Nakazawaea wickerhamii* 1885, Wa 2032: *Wickerhamomyces anomalus* 2032, Wa 2033: *Wickerhamomyces anomalus* 2033.

3.2.4. Cholesterol assimilation

The ability of the assayed oil-borne yeast species and the probiotic reference yeast, *S. boulardii*, to remove *in vitro* cholesterol in three different media was tested. The results revealed that the percentage of cholesterol reduction depends on the oil-borne yeast strain and the type of culture medium used (Table 4). The medium where all yeast strains studied showed the highest percentage of cholesterol removal was YNB containing cholesterol and glucose (CG), with values ranging from 3.15% (*C. adriatica* 1985) to 62.73% (*W. anomalus* 2032). When YNB was supplemented with cholesterol, glucose and ox gall (CGO), a decrease in cholesterol assimilation was recorded in all yeasts, with the exception of *C. adriatica* 2036 and *W. anomalus* strains 2032 and 2033, with values ranging from 0.80% (*C. diddensiae* 1918) to 63.71% (*W. anomalus* 2032), with significant differences between the extreme values. In contrast, the medium with the lowest percentage of cholesterol removal was YNB supplemented with cholesterol and ox gall (CO). Here, the percentage of cholesterol-reduction ranged between 0.00% (*C. adriatica* 1985, *C. diddensiae* strains 1918 and 1922, and *Y. terventina* strains 1987 and 1988) and 42.11% (*W. anomalus* 2033). This behavior can be explained by the toxic effect of ox gall on some oil-borne yeasts, including the reference yeast, *S. boulardii*. The toxic effect of ox gall on yeast cells was higher when glucose was missing in the medium (Table 4). The results obtained from the *W. anomalus* strains 2032 and 2033, *N. molendini-olei* strains 1926 and 2035, and *C. adriatica* 2036 were similar to those obtained from the same yeast species isolated from table olives (Porru et al., 2018). Different results were recorded for *C. diddensiae* strains 1918 and 1922 and *S. boulardii*, which failed to metabolize cholesterol in the CO medium (cholesterol and ox gall) lacking glucose. These differences can be attributed to the different original habitats (olive oil, table olives) or, in the case of *S. boulardii*, to the different commercial sources (Ultra Levura, Zambon S.A.U, Barcelona, Spain or Zambon Italia, s.r.l., Bresso-Milan, Italy). The cluster analysis of the yeast strains, based on their abilities to metabolize cholesterol in all the media used (CG, CGO and CO), showed two clusters (Fig. 2). In the first cluster, the oil-borne yeast strains with a cholesterol-removing capacity similar to that of the reference yeast *S. boulardii* were grouped, while the more promising oil-borne yeasts characterized by a better performance represented by *C adriatica* 2036, *N. molendini-olei* strains 1926 and 2035, *N. wickerhamii* 1885 and *W. anomalus* strains 2032 and 2033 were grouped (Fig. 2). Among the more promising yeast strains studied, both the *W. anomalus* 2032 and 2033 strains of the same cluster showed the highest cholesterol-removal capacity in all the media used. The cholesterol-removal activity of these two yeast strains varied between a minimum of 36.28% (CO) to a maximum of 62.73% (CG) and by a minimum of 42.11% (CO) to a maximum of 58.57% (CG), respectively. The data listed in Table 4 place the cholesterol-removal capacity of the *W. anomalus* strains at the same level of some lactic acid bacteria (LAB), isolated from dairy and non-dairy sources, characterized by a high cholesterol-removal capacity equal to 43.7% (Shehata et al., 2016). Accordingly, owing to the good probiotic traits of some of the oil-borne yeast strains studied, their presence in virgin olive oil, as a component of the oily microbiota, can have beneficial effects in the long run and potentially can be used in functional foods in which cholesterol reduction is the main target.

4. Conclusions

In this research, we studied the physiological characteristics and the potential *in vitro* probiotic features of eleven oil-borne yeast strains belong to *Y. terventina*, *C. diddensiae*, *C. adriatica*, *N. molendini-olei*, *N. wickerhamii*, and *W. anomalus* species isolated from virgin olive oil. The results obtained from the *in vitro* analysis of their multifunctional activities showed that olive oil-borne yeasts may have a healthy potential with interesting probiotic features. Unlike *S. boulardii*, that do not

Table 5
Physiological characteristics of yeast strains isolated from virgin olive oil.

Yeast strains	Hydrophobicity (%)	Autoaggregation capacity (%)	Biofilm assay $(OD)_{595}$	Decarboxylation activity
Sbo	26.00 (8.49)^{b}	43.44 (10.48)^{a}	0.03 (0.01)^{c}	–
Yt 1987	45.50 (0.71)^{a}	33.33 (11.99)^{b}	0.86 (0.10)^{a}	–
Yt 1988	37.50 (3.53)^{ab}	29.73 (10.29)^{b}	0.85 (0.09)^{a}	–
Cd 1918	16.50 (0.71)^{c}	23.33 (7.85)^{bc}	0.18 (0.03)^{bc}	–
Cd 1922	41.50 (6.36)^{ab}	47.50 (14.14)^{a}	0.54 (0.06)^{b}	–
Ca 1985	55.50 (6.36)^{a}	32.00 (8.49)^{b}	0.60 (0.11)^{b}	–
Ca 2036	19.50 (3.53)^{c}	10.00 (2.83)^{c}	0.07 (0.01)^{c}	–
Nm 1926	0.00	28.00 (8.49)^{b}	0.05 (0.02)^{c}	–
Nm 2035	0.00	8.33 (2.20)^{c}	0.03 (0.01)^{c}	–
Nw 1885	0.00	18.17 (4.23)^{bc}	0.57 (0.12)^{b}	–
Wa 2032	24.00 (1.41)^{bc}	31.63 (9.44)^{b}	0.67 (0.15)^{ab}	+
Wa 2033	20.50 (3.53)^{c}	27.78 (8.09)^{b}	0.76 (0.21)^{a}	+

Data are reported as mean and standard deviation (in parenthesis) obtained from triplicate trials. Different letter in the same column indicate significant differences by Tukey's test ($P < 0.05$). Sbo: *Saccharomyces boulardii* (reference yeast), Yt 1987: *Yamadazyma terventina* 1987, Yt 1988: *Yamadazyma terventina* 1988, Cd 1918: *Candida diddensiae* 1918, Cd 1922: *Candida diddensiae* 1922, Ca 1985: *Candida adriatica* 1985, Ca 2036: *Candida adriatica* 2036, Nm 1926: *Nakazawaea molendini-olei* 1926, Nm 2035: *Nakazawaea molendini-olei* 2035, Nw 1885: *Nakazawaea wickerhamii* 1885, Wa 2032: *Wickerhamomyces anomalus* 2032, Wa 2033: *Wickerhamomyces anomalus* 2033. +, positive decarboxylation activity; -, negative decarboxylation activity.

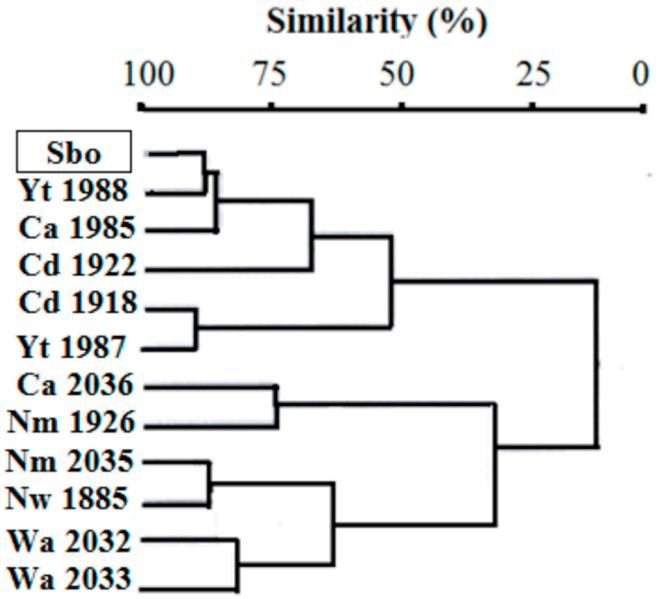

Fig. 2. Global cluster analysis of the yeast strains able to metabolize cholesterol in the GC, CGO, and CO media. (Sbo: *Saccharomyces boulardii* (reference yeast), Ca 1985: *Candida adriatica* 1985, Ca 2036: *Candida adriatica* 2036, Cd 1918: *Candida diddensiae* 1918, Cd 1922: *Candida diddensiae* 1922, Nm 1926: *Nakazawaea molendini-olei* 1926, Nm 2035: *Nakazawaea molendini-olei* 2035, Nw 1885: *Nakazawaea wickerhamii* 1885, Wa 2032: *Wickerhamomyces anomalus* 2032, Wa 2033: *Wickerhamomyces anomalus* 2033, Yt 1987: *Yamadazyma terventina* 1987, Yt 1988: *Yamadazyma terventina* 1988).

produced PUFAs, olive oil-borne yeast strains synthesize PUFAs with two and three double bonds in amounts superior to those found in olive oil. The most representative PUFAs are linoleic acid (omega-6 fatty acid) and linolenic acid (omega-3 fatty acid), which are two essential fatty acids that may have beneficial health effects in the long run. The trials carried out on the resistance to gastric and pancreatic juices, demonstrated for first time that, in the case of the yeast cells that were coated with olive oil (a condition similar to that of the common yeasts in virgin olive oil used by consumers), the survival of all yeasts studied, including *S. boulardii,* increased considerably, reaching a maximum of 100%. Additionally, cholesterol was removed *in vitro* by 50% of the oil-borne yeast strains which represent an important probiotic trait. The best results were recorded for the *W. anomalus* species. Further *in vitro* and *in vivo* experiments are underway to confirm the observed properties, such as the synthesis of beneficial PUFAs and the hypocholesterolemic effect of some olive oil-borne yeasts.

References

Avery, S.V., Howlett, N.G., Radice, S., 1996. Copper toxicity towards *Saccharomyces cerevisiae*: dependence on plasma membrane fatty acid composition. Appl. Environ. Microbiol. 62 (11), 3960–3966.

Bassam, A., Anwar, A., Sabah, Y., 2014. Identification of some yeasts by API 20C system and fatty acid profile. Adv. Environ. Biol. 8 (5), 1260–1266.

Bonatsou, S., Benitez, A., Rodriguez-Gomez, F., Panagou, E.Z., Arroyo-Lopez, F.N., 2015. Selection of yeasts with multifunctional features for application as starters in natural black table olive processing. Food Microbiol. 46, 66–73.

Čadež, N., Raspor, P., Turchetti, B., Cardinali, G., Ciafardini, G., Veneziani, G., Péter, G., 2012. *Candida adriatica* sp. nov. and *Candida molendinolei* sp. nov., two yeast species isolated from olive oil and its by-products. Int. Syst. Evol. Microbiol. 62, 2296–2302.

Ciafardini, G., Zullo, B.A., 2002. Microbiological activity in stored olive oil. Int. J. Food Microbiol. 75, 111–118.

Ciafardini, G., Zullo, B.A., 2015. Effect of lipolytic activity of *Candida adriatica, Candida diddensiae* and *Yamadazyma terventina* on the acidity of extra-virgin olive oil with different polyphenol and water content. Food Microbiol. 47, 12–20.

Ciafardini, G., Zullo, B.A., 2018. Virgin olive oil yeasts: a review. Food Microbiol. 70, 245–253.

Ciafardini, G., Cioccia, G., Peca, G., Zullo, B.A., 2004. Transfer of selected yeasts to oil through olive inoculation. Ital. J. Food Sci. 1, 1–7.

Ciafardini, G., Zullo, B.A., Cioccia, G., Iride, A., 2006a. Lipolytic activity of *Williopsis californica* and *Saccharomyces cerevisiae* in extra virgin olive oil. Int. J. Food Microbiol. 107, 27–32.

Ciafardini, G., Zullo, B.A., Iride, A., 2006b. Lipase production by yeasts from extra virgin olive oil. Food Microbiol. 23, 60–67.

Ciafardini, G., Cioccia, G., Zullo, B.A., 2013a. Survival of *Candida parapsilosis* yeast in olive oil. Ann. Microbiol. 63, 1645–1648.

Ciafardini, G., Zullo, B.A., Antonielli, L., Corte, L., Roscini, L., Cardinali, G., 2013b. *Yamadazyma terventina* sp. nov., a yeast species of the *Yamadazyma* clade from Italian olive oils. Int. Syst. Evol. Microbiol. 63, 372–376.

Ciafardini, G., Cioccia, G., Zullo, B.A., 2017. Taggiasca extra virgin olive oil colonization by yeasts during the extraction process. Food Microbiol. 62, 58–61.

Czerucka, D., Piche, T., Rampal, P., 2007. Review article: yeast as probiotics –*Saccharomyces boulardii*. Aliment. Pharmacol. Ther. 26 (6), 767–778.

EC, 2002. European Commission Regulation No. 796/2002 of 6 May 2002, Amending Regulation (EEC) No. 2568/91 on the Characteristics of Olive Oil and Olive-pomace Oil and the Relevant Methods of Analysis and the Additional Notes in the Annex to Council Regulation (EEC) No. 2658/87 on the Tariff and Statistical Nomenclature and on the Common Customs Tariff.

EC, 2013. European Commission Regulation No. 299/2013 of 26 March 2013 Amending Regulation (EEC) No. 2561/91 on the Characteristics of Olive Oil and Olive Residue Oil and on the Relevant Methods of Analysis.

Eder, K., 1995. Gas chromatographic analysis of fatty acid methyl esters. J. Chromatogr. B Biomed. Sci. Appl. 671 (1–2), 113–131.

Food and Agriculture Organization of the United Nations and World Health Organization, 2001. Expert Consultation Report. FAO/WHO, Geneva, Switzeland (Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria).

García-Cayuela, T., Korany, A.M., Bustos, I., Gomez de Cadinanos, L.P., Requena, T., Peláez, C., Martínez-Cuesta, M.C., 2014. Adhesion abilities of dairy Lactobacillus plantarum strains showing an aggregation phenotype. Food Res. Int. 57, 44–50.

Gardini, F., Tofalo, R., Belletti, N., Iucci, L., Suzzi, G., Torriani, S., Guerzoni, M.E., Lanciotti, R., 2006. Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. Food Microbiol. 23, 641–648.

Gil-Rodríguez, A.M., Carrascosa, A.V., Requena, T., 2015. Yeasts in food and beverage: *in vitro* characterization of probiotic traits. LWT-Food Sci. Techonol. 64 (2), 1156–1162.

Glaser, C., Demmelmair, H., Koletzko, B., 2010. High-throughput analysis of total plasma fatty acid composition with direct *in situ* transesterification. J. Lipid Res. 51 (2), 216–221.

Justino, C.I.L., Pereira, R., Freitas, A.C., Rocha-Santos, T.A.P., Panteleitchouk, T.S.L., Duarte, A.C., 2012. Olive oil mill wasterwaters before and after treatment: a critical review from the ecotoxicological point of view. Ecotoxicology 21, 615–619.

Kalai Chelvam, K., Chai, L.C., Thong, K.L., 2014. Variations in motility and biofilm formation of *Salmonella enterica* serovar Typhi. Gut Pathog. 6, 2.

Kock, J.L.F., Botha, A., 1998. Fatty acids in fungal taxonomy. In: Frisvad, J.C., Bridge, P.D., Arora, D.K. (Eds.), Chemical Fungal Taxonomy. Marcel Dekker, New York, pp. 219–246.

Kourelis, A., Kotzamanidis, C., Litopoulou-Tzanetaki, E., Scouras, Z.G., Tzanetakis, N., Yiangou, M., 2010. Preliminary probiotic selection of dairy and human yeast strains. J. Biol. Res. (Thessalon.) 13, 93–104.

Kurtzman, C.P., Fell, J.W., 1998. The Yeasts, a Taxonomic Study, fourth ed. Elsevier, Amsterdam, pp. 1055.

Kurtzman, C.P., Robnett, C.J., 1997. Identification of clinical important ascomycetous yeast based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. 5, 1216–1223.

Kutty, S.N., Philip, R., 2008. Marine yeasts – a review. Yeast 25, 465–483.

McFarland, L.V., Bernasconi, P., 1993. *Saccharomyces boulardii*: a Review of an innovative biotherapeutic agent. Microb. Ecol. Health Dis. 6, 157–171.

Oliveira, T., Ramalhosa, E., Nunes, L., Pereira, J.A., Colla, E., Pereira, E.L., 2017. Probiotic potential of indigenous yeasts isolated during the fermentation of table olives from Northeast of Portugal. Innovat. Food Sci. Emerg. Technol. 44, 167–172.

Ouwehand, A.C., Salminen, S., Isolauri, E., 2002. Probiotics: an overview of beneficial effects. Antonie Leeuwenhoek 82 (1–4), 279–289.

Petroni, A., Blasevich, M., Salami, M., Papini, N., Montedoro, G.F., Galli, C., 1995. Inhibition of platelet aggregation and eicosanoid production by phenolic component of olive oil. Thromb. Res. 78, 151–160.

Porru, C., Rodríguez-Gómez, F., Benítez-Cabello, A., Jiménez-Díaz, R., Zara, G., Budroni, M., Mannazzu, I., Arroyo-López, F.N., 2018. Genotyping, identification and multifunctional features of yeasts associated to Bosana naturally black table olive fermentations. Food Microbiol. 69, 33–42.

Psani, M., Kotzekidou, P., 2006. Technologic characteristics of yeast strains and their potential as starters adjuncts in Greek-style black olive fementation. World J. Microbiol. Biotechnol. 22, 1329–1336.

Romo-Sánchez, S., Alves-Baffi, M., Arévalo-Villena, M., Úbeda-Iranzo, J., Briones-Pérez, M., 2010. Yeast biodiversity from oleic ecosystems: study of their biotechnological properties. Food Microbiol. 27, 487–492.

Rosenberg, M., 2006. Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. FEMS Microbiol. Lett. 262, 129–134.

Santona, M., Sanna, M.L., Multineddu, C., Fancello, F., de la Fuente, S.A., Dettori, S., Zara, S., 2018. Microbial biodiversity of Sardinian oleic ecosystems. Food Microbiol. 70, 65–75.

Shehata, M.G., El Sohaimy, S.A., El-Sahn, M.A., Youssef, M.M., 2016. Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. Ann. Agric. Sci. 61 (1), 65–75.

Shen, Y.B., Piao, X.S., Kim, S.W., Wang, L., Liu, P., Yoon, I., Zhen, Y.G., 2009. Effects of yeast culture supplementation on growth performance, intestinal health, and immune response of nursey pigs. J. Anim. Sci. 87, 2614–2624.

Silva, T., Reto, M., Sol, M., Peito, A., Peres, C.M., Xavier-Malcata, F., 2011. Characterization of yeasts from Portuguese brined olives, with a focus on their potentially probiotic behavior. LWT-Food Sci. Technol. 44, 1349–1354.
Syal, P., Vohra, A., 2013. Probiotic potential of yeast from traditional Indian fermented foods. Int. J. Microbiol. Res. 5 (2), 390–398.
Torrea Goni, D., Aucìn Azpilicueta, C., 2001. Influence of yeast strain on biogenic amines content in wine: relationship with the utilization of amino acid during fermentation. Am. J. Vitic. 62, 185–190.
Visioli, F., Bellomo, G., Montedoro, G.F., Galli, C., 1995. Low-density lipoprotein oxidation is inhibited *in vitro* by olive oil constituents. Atherosclerosis 117, 25–32.
Wenzhou, L.V., Abd El-Latif, H., Yu, H., Xinchun, L., Min, Y., 2011. Impacts of cell surface characteristics on population dynamics in a sequencing batch yeast reactor treating vegetable oil-containing wastewater. Appl. Microbiol. Biotechnol. 90, 1785–1793.
Yazawa, H., Iwahashi, H., Kamisaka, Y., Kimura, K., Uemura, H., 2009. Production of polyunsatured fatty acids in yeast *Saccharomyces cerevisiae* and its relation to alkaline pH tolerance. Yeast 26 (3), 167–184.
Zullo, B.A., Ciafardini, G., 2008. Lipolytic yeasts distribution in commercial extra virgin olive oil. Food Microbiol 25, 970–977.
Zullo, B.A., Cioccia, G., Ciafardini, G., 2010. Distribution of dimorphic yeast species in commercial extra virgin olive oil. Food Microbiol 27, 1035–1042.