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# Microbial biodiversity of Sardinian oleic ecosystems

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ABSTRACT

The olives are rich in microorganisms that, during the extraction process may persist in the oils and can influence their physicochemical and sensory characteristics. In this work, and for the first time, we isolated and identified microbial species, yeast and bacteria, present during the production process in four Sardinian (Italy) oleic ecosystems. Among these varieties, we found that *Nera di Gonnos* was associated to the highest microbial biodiversity, which was followed by *Bosana, Nocellara del Belice* and *Semidana.* Among the different microbial species isolated, some are specific of olive ecological niches, such as *Cryptococcus* spp and *Serratia* spp; and others to olive oils such as *Candida* spp and *Saccharomyces*. Some other species identified in this work were not found before in oleic ecosystems. The enzymatic analyses of yeast and bacteria showed that they have good β-glucosidase activity and yeast also showed good β-glucanase activity. The majority of bacteria presented lipolytic and catalase activities while in yeast were species-specific. Interestingly, yeast and bacteria isolates presented a high resistance to bile acid, and about 65% of the yeast were able to resist at pH 2.5 for 2 h. Finally, bacteria showed no biofilm activity compared to yeast.



## 1. Introduction

Olives are rich in microorganisms that during the extraction process can be maintained in the olive oil, and, according to their metabolic activities, may affect olive oil sensorial and physicochemical properties. Despite this, olive oil microbiological characteristics has been poorly analyzed in the past, even if their role in oil lipolysis was already hypothesized (Tassou et al., 2010).

However, in the last 15 years, several researchers found that olive oil is rich in microflora (Ciafardini and Zullo, 2002; Ciafardini et al., 2006; Zullo and Ciafardini, 2008; Romo-Sanchez et al., 2010; Zullo et al., 2010; Mari et al., 2016; Ciafardini et al., 2017) that can impact positively or negatively on the physical-chemical and sensory qualities of the oils. The presence of these microorganisms, particularly yeasts, is due to their migration from olive carposphere to olive oil during the extraction process. Some microorganisms do not survive long time, but others may persist and be metabolically active during the storage period and become a typical microbiota of olive oil (Zullo et al., 2010). Moreover, it has been shown that oils from microbiologically contaminated olives contain lower amounts of C5 volatiles, chlorophylls, pheophytins and xanthophylls, which are responsible for their sensory characteristics (Vichi et al., 2011).

The most common yeast species found in the extra virgin olive oil microbial flora belong to *Candida diddensiae*, *Candida boidinii*, *Candida wickerhamii*, *Williopsis californica, Candida guilliermondi*i, *Candida parapsilosis, Candida adriatica*, *Candida molendiniolei* and *Saccharomyces cerevisiae* (Ciafardini et al., 2006; Cadez et al., 2012). These yeast species can produce enzymes (Ciafardini and Zullo, 2002; Ciafardini et al., 2006b), throughout the entire preservation period, therefore the quality of olive oil may change at any time and according to the predominant yeast species.

Olive oils taste can be improved by the β-glucosidase and esterase producing species of yeasts, which are capable of hydrolyzing the oleuropein into simpler and no longer bitter compounds characterized by a high antioxidant activity (Ciafardini and Zullo, 2002). However, the presence of some lipase producing yeasts can worsen olive oil quality through triglycerides hydrolysis. For example (Ciafardini and Zullo, 2015), found that the genus *Candid*a has a negative effect on the oil quality, in particular *C. adriatica,* due to its enzymatic hydrolysis of the triacylglycerols. In addition, the presence of dimorphic yeast forms like the opportunistic pathogen species *C. parapsilosis* and *C. guilliermondii* recently observed by Zullo et al. (2010), can be considered potentially dangerous for the human health.

The purpose of this preliminary study was to isolate, identify and characterize microbial species, yeast and bacteria, present during the production process (from olives to olive paste, and to oils) of extra virgin olive oils produced in Sardinia (Italy) from olives belonging to *Nera di Gonnos*, *Nocellara del Belice*, *Semidana* and *Bosana* "local" varieties.

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## 2. Materials and methods

### 2.1. Isolation of yeast and bacteria

The microorganisms were isolated from olives, olive paste and oils samples. Samples were obtained during harvest time (November–January 2014/2015) from the north Sardinia region (Italy). Harvested or collected samples were transported to the laboratory and refrigerated under aseptic conditions.

For olives sampled just before the milling process, 50 g were put under agitation for 1 h in 250 ml Erlenmeyer flasks filled with 100 ml of Ringer's solution (Oxoid). For the olive pastes, aliquots of 10 g of olive paste were mixed manually with 90 ml of Ringer's solution for 10 min in sterile bags. For oils, sampled just after the milling process, 10 ml of oil was mixed with 90 ml of YEPD (1% yeast extract, 2% peptone, 2% glucose) in 250 ml Erlenmeyer flanged flasks. The samples were then put under agitation for 48 h at 30 °C.

For the subsequent isolation, 100 μL from all samples (olives, olive paste and oils) were taken, and 1/10 dilutions were plated onto YEPD and PCA (Plate Count Agar, Oxoid) plates. The plates were left to incubate at 30 and 37 °C for 48 h. Randomly selected colonies, representative of the different colony morphologies (shape, color, dimension etc.), were picked up, re-streaked on PCA and stored at −80 °C.

### 2.2. DNA isolation

Around 80 colonies between yeast and bacteria, previously selected, were inoculated into 15 ml sterile polypropylene tubes containing 7 ml of YEPD and kept in overnight at 30 °C in agitation. The following day the samples were centrifuged for 2 min at 3500 rpm and the supernatant was eliminated. The obtained pellet was resuspended in 500 μl of distilled water ($H_2O$) and transferred into 1.5 ml Eppendorf tubes and centrifuged again at 14,000 rpm for 5 s. After eliminating the new supernatant, the pellet was resuspended in the residual liquid and 200 μl of sol. Then, a solution of 2% Triton X-100, 1% SDS 10% solution, 100 mM NaCl, 10 mM Tris-HCl, 1 mM Na 2 EDTA), 200 μl of phenol-chloroform isoamyl alcohol 25: 24: 1) and 0.3 g of glass beads solution (Oxoid) were added to the pellet solution. The samples were vortexed for 2 min to facilitate breaking of the cell walls, added with to 200 μl of TE pH 8 (10 mM Tris-HCL, 1 mM Na2 EDTA) and centrifuged for 5 min at 14,000 rpm. After centrifugation the upper phase was transferred to new 1.5 ml Eppendorf tubes and the DNA was precipitated by adding 800 μl of 100% ethanol and centrifuged for 3 min at 14,000 rpm. Subsequently the DNA was washed with 800 μl of 70% ethanol and then centrifuged again. The supernatant was discarded and the pellet was air-dried. After drying the pellet (now mostly composed by DNA), was resuspended in 50 μl of $H_2O$, centrifuged, and 45 μl transferred to a new 1.5 ml Eppendorf tube and stored at −20 °C for the subsequent analyses.

### 2.3. Molecular characterization and identification of isolates

*Yeast*. Molecular characterization of isolates was conducted by means of polymerase chain reaction of two Internal Transcribed Spacers (ITS1 and ITS4) of 5.8 rRNA by using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) ed ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′), as described by Esteve-Zarzoso et al. (1999).

The amplification protocol was performed using a thermocycler (MyCycler Thermal Cycler System, Biorad). Temperature cycling parameters were as follows: an initial cycle of 15 min at 95 °C, and 40 cycles as follows: 30 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C. Ampicons were visualized using the Chemidoc XRS system (Biorad, Carlsbad, CA, USA). The amplified products obtained were purified and sequenced at the BMR genomics (Padova) and Macrogen (Hong Kong, China).

*Bacteria*. Isolated strains were subjected at 16S rDNA sequencing. The DNA of single strain was extracted as reported in section 2.2 and the 16S ribosomal DNA fragment (1500 bp) was amplified using the universal primers W001 (5′-AGAGTTTGATCMTGGCTC-3′) and W002 (5′-GNTACCTTGTTACGACTT-3′) as previously described by Godon et al. (1997). Subsequently, amplicons were sequenced after being purified with QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. Sequencing with primer W001 and W002 was performed at Macrogen (Hong Kong, China). An average of 800 bp *per* sequence were obtained and compared with those presents in the GenBank database using the BLAST program (http://www.ncbi.nih.gov/BLAST/), and with those in the Ribosomal Database project (http://rdp.cme.msu.edu/edu/index.jsp). All data related to the BLAST analyses are summarized in Table S1. Degree of biodiversity was also assessed using Past3 (PAST PAleontological STatistics Version 3.16). The utilization of this software confirmed the good level of microbial species diversity in all the oleic ecosystems analyzed (Table S2).

### 2.4. Enzymatic characterization of isolates

β-glucosidase screening was carried out on agar plates with arbutin as substrate. The medium consisted of 6.7 g/L Yeast Nitrogen Base (YNB; Difco), 5 g/L arbutin (Sigma), 20 g/L agar. The pH was adjusted to 5.0. The medium was autoclaved at 121 °C for 15 min. Immediately after sterilization 2 ml of a sterile 1% (w/v) ferric ammonium citrate solution was added to 100 ml of medium, and then poured into Petri dishes (15 ml of medium *per* plate). Each plate was inoculated with 8 different isolates, incubated at 25 °C and examined after 2, 4, 6 and 8 d. A non-inoculated plate served as the control. Strains with β-glucosidase activity hydrolyzed the substrate and a dark brown color developed in the agar (Rosi et al., 1994). For its analysis a code based on the color of the halo was assessed: white: 0; light grey: 1; grey: 2 and black: 3.

Production of β-glucanase activity was determined by streaking the isolates onto YPD plates containing 0.2% lichenan (Sigma). The plates were incubated for 5 d at 30 °C. Then, colonies were rinsed off the plates with distilled water and plates stained with 0.03% Congo Red. A clear zone around the colony identified β–glucanase activity (Strauss et al., 2001). For the analysis a code based on the diameter of the halo was assessed with numbers 0, 1 and 2.

To assess the ability of the isolates to decarboxylate amino acids, by producing biogenic amines, a specific media has been used. Aliquots of 0.1 g/l of glucose, 0.06 g/l of Bromocresol purple (Sigma-Aldrich) and 1 g/l of each amino acid (L-lysine, L-phenylalanine, L-tyrosine, L-histidine, L-arginine) together and adding the single above-mentioned amino acid to the medium. 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, were aseptically added. Final pH was adjusted to 5.3 using HCl. Isolates were streaked on the agar plates and then incubated at 25 °C for 4 d. The reaction was considered positive if a violet halo appeared around the colonies (Gardini et al., 2006). In this case a (+) or (−) code was assessed based on the presence or absence of the surrounding halo.

For the catalase activity, the method proposed by Whittenbury (1964) was slightly modified. Isolates were inoculated overnight in liquid YPD medium in 96-wells plates. The following day, catalase activity was evaluated by adding 3% (v/v) of hydrogen peroxide to the inocula. Presence or absence of bubbles was indicative of catalase activity, and assessed with (+) or (−) code.

Isolates were evaluated in terms of their lipase activities on Spirit Blue agar (Sigma-Aldrich, St. Louis, USA) which includes the pancreatic digest of casein (1%), yeast extract (0.5%), agar (1.7%) and spirit blue (0.015%). Lipase substrate mix contains Twin 80 (cat. 93,780) and olive oil. Lipase substrate was added to the medium following manufactory instructions. Plates were inoculated with the same amounts of cell suspensions and incubated at 25 °C for 48 h. The lipolysis was assessed by observation of halos on the plate indicating that microorganisms metabolized the lipids. All enzymatic tests were performed in triplicate.

### *2.5. Biofilm and mat formation*

Biofilm and mat formation were evaluated as previously described (Reynolds and Fink, 2001), with some modifications as described by Bou Zeidan et al. (2014). Aliquots of 100 μL of cell suspensions containing $5 \times 10^6$ cells $ml^{-1}$ in SD (Synthetic Dextrose) medium were dispensed into 96-well polystyrene microtiter plates (Costar 3595, Corning, NY). Cell suspensions were incubated statically at 30 °C for 48 h. Then, an equal volume of 1% (w/v) crystal violet was added to each well. After 30 min, the wells were washed with sterile water, and the adherence of cells was quantified by solubilizing the retained crystal violet in 100 μL of 96% ethanol. After 30 min, 50 μL of these solutions were transferred to fresh 96-well polystyrene microtiter plates (Corning), and then $A_{570}$ was measured spectrophotometrically. Only isolates with a $A_{570}$ value over 1.4 were considered positives. For the mat formation on soft agar, strains were inoculated onto YPD soft agar plates (0.3% agar) with a toothpick 1–2 days after the plates were poured, as previously described (Reynolds and Fink, 2001). The plates were wrapped with Parafilm, incubated at 25 °C for 21 days and then photographed.

### *2.6. Screening for tolerance to acidic pH and bile*

For the acidic pH, strains were grown in 24-wells plates in YPD broth at 25 °C overnight, 10 (7) cells/mL of each active cultures were inoculated in 24-wells plates containing 1 mL of YPD broth adjusted to pH 2.5 with 12 N HCl and incubated at 37 °C for 2 h. Samples were taken after 2 h and the viable strains were identified by plating 10 μL of cell cultures on YPD media and incubating the plates 24 and 48 h at 25 °C.

For the bile tolerance test, strains were grown in 24-wells plates in YPD broth at 25 °C overnight, 3% saturated bile solution was prepared separately by dissolving powdered bile salts (Fluka cod. #48305) in YPD broth. Bile solution was then filter sterilized by 0.2 μm filter. Bile solution was added into 24-wells plates containing 1 mL of YPD broth to achieve a final concentration of 0.3, 0.75 and 1.5%. Wells with 0% of bile solution served as a control samples. A number of $10^7$ cells/mL of each active cultures were inoculated into the 24-wells plates previously described. The cultures were incubated at 37 °C for 2 h. Samples were taken after 2 h and the viable strains were identified by plating 10 μL of cell cultures on YPD media and incubating the plates 24 and 48 h at 25 °C. All the experiments were performed in triplicate.

## 3. Results and discussion

Previous studies (Ciafardini et al., 2017) revealed that the olive oil microbiota might play an important role in the definition of the sensory characteristics. Furthermore, since the increase of titratable acidity of olive oil during storage is attributed to enzymatic activity rather than autocatalysis, the role of microorganisms found in olive oil should be further investigated (Tassou et al., 2010).

In this work, through the isolation, molecular identification, characterization of microorganisms obtained during the production process of different extra virgin olive oils produced in Sardinia (Italy), it was possible to define yeast and bacteria microbiota of different oleic ecosystem varieties. Total bacterial count was estimated using two different non selective media (YEPD and PCA) for both yeast and bacteria. The microbial counts of the different oleic ecosystems (identified by each individual olive variety) showed a concentration of microorganisms of about $1.5*10^4$ CFU/ml in olives and $1.5*10^4$ CFU/ml in olive paste for Bosana; about $3.5*10^4$ CFU/ml in olives and about $1.5*10^4$ CFU/ml olive paste for Semidana; about $2.6*10^4$ CFU/ml in olives and about $2.5*10^4$ CFU/ml in olive paste in Nera di Gonnos; and finally, about $2*10^4$ CFU/ml in olives and $3*10^3$ CFU/ml in olive paste for Nocellara del Belice. Isolation of oil microflora was done using the enrichment method so the estimation of the concentration was not a real estimation of the CFU/ml effectively present in the oils. Anyhow, CFU/ml concentrations of olive oils subjected to enrichment was always about $2*10^3$ for all the oils tested. The first observation has been the strong selective pressure of the isolates from olives to oils, as it is possible to observe in Table 1. This data reinforced what recently found by Ciafardini et al. (2017), about the strong selective pressure on yeast in the mono varietal olive oils. The good level of microbial species diversity in all the oleic ecosystems was also assessed (Table S2).

### *3.1. Molecular characterization*

*Yeast*. The yeasts isolated from different oleic ecosystems in Sardinia belong mainly to *Candida genus,* as already observed by Ciafardini et al. (2006), but also *Cryptococcus* genus. Amplicons of around 500bp-800bp, belonging to the ITS region, were obtained by PCR and then sequenced. The yeast genera and/or species were identified after screening for DNA homology using the NCBI Blast program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). About 20 different species belonging to nine different genera of yeast were identified. In particular, the following genus were found: *Cryptococcus, Aureobasidium, Debaryomyces, Rhodotorula, Pirula* and *Candida* in olives; *Saccharomyces, Nakazawaea, Can*dida and *Pichia* in olive paste; and finally *Candida*, *Saccharomyces* and *Pichia* in the oils (Table 1).

*Bosana* with 12 species showed the highest richness, followed by *Nera di Gonnos* variety (10 species) Semidana (8 species) and *Nocellara del Belice* (7 species). For all analyzed varieties, a broader biodiversity was found in olives which decreases from the olive paste during malaxation to olive oil. The species found in the olives were 15: *Candida* spp, (4 species), *Cryptococcus* spp (4 species), *S. cerevisiae*, *Aureobasidium pullulans*, *Rhodotorula glutinis, Nakazawaea anatomiae*, *Rhodosporidium babievae, Pirula salina*. The species found in olive paste were 9: *Candida* spp (four species), *S. cerevisiae*, *Cryptococcus carnescens*, *Pichia manshurica and N. anatomiae;* and finally the species found in the olive oils were 6: *Candida* spp (three species) *S. cerevisiae, Pichia Mexicana* and *Pichia nakazawaea*. *Candida dendronema* were found only in the olive oils. *C. adriatica* was the only species identified in *Semidana* olive oil and in four olive oil

**Table 1**
Yeast Isolates divided *per* species and olive varieties.

| Yeasts species | N° isolates | Olive Varieties | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Bosana* | | | *Semidana* | | | *Nera di Gonnos* | | | *Nocellara del Belice* | | |
| | | Olive | Paste | Oil | Olive | Paste | Oil | Olive | Paste | Oil | Olive | Paste | Oil |
| *Candida molendinolei* | 11 | – | 3 | – | 2 | – | – | – | 1 | – | – | 5 | – |
| *Candida adriatica* | 7 | – | 2 | – | 1 | – | 3 | – | 1 | – | – | – | – |
| *Saccharomyces cerevisiae* | 6 | – | 2 | – | – | 2 | – | – | – | 1 | – | 1 | – |
| *Cryptoccoccus carnescens* | 5 | 1 | – | – | – | – | – | – | – | – | 4 | – | – |
| *Aureobasidium pullulans* | 5 | 3 | – | – | 1 | – | – | 1 | – | – | – | – | – |
| *Rhodotorula glutinis* | 3 | 3 | – | – | – | – | – | – | – | – | – | – | – |
| *Candida wickerhami* | 3 | – | – | – | – | – | – | – | – | – | – | 3 | – |
| *Pichia manshurica* | 2 | – | – | – | – | – | – | – | 2 | – | – | – | – |
| *Nakazawaea anatomiae* | 2 | – | – | – | 1 | – | – | – | 1 | – | – | – | – |
| *Candida temnochilae* | 2 | – | – | – | – | – | – | – | 1 | 1 | – | – | – |
| *Cryptococcus magnus* | 1 | 1 | – | – | – | – | – | – | – | – | – | – | – |
| *Pichia mexicana* | 1 | – | – | – | – | – | – | – | – | – | – | – | 1 |
| *Rhodosporium babjevae* | 1 | – | – | – | 1 | – | – | – | – | – | – | – | – |
| *Candida diddensiae* | 1 | – | – | – | – | – | – | – | 1 | – | – | – | – |
| *Candida guilliermondii* | 1 | – | – | – | – | – | – | 1 | – | – | – | – | – |
| *Cryptococcus oeirensis* | 1 | 1 | – | – | – | – | – | – | – | – | – | – | – |
| *Cryptococcus victoriae* | 1 | 1 | – | – | – | – | – | – | – | – | – | – | – |
| *Candida dendronema* | 1 | – | – | – | – | – | – | – | – | – | – | – | 1 |
| *Pirula Salina* | 1 | 1 | – | – | – | – | – | – | – | – | – | – | – |
| *Pichia nakazawaea* | 1 | – | – | – | – | – | – | – | – | 1 | – | – | – |
| *Candida* sp *CLIB 1308* | 1 | – | – | – | 1 | – | – | – | – | – | – | – | – |
| *Rhodororula* spp | 1 | 1 | – | – | – | – | – | – | – | – | – | – | – |
| *Others* | 6 | 2 | – | – | 2 | – | – | 1 | – | – | – | 1 | – |
| **Total** | **64** | **14** | **7** | **-** | **9** | **2** | **3** | **3** | **7** | **3** | **4** | **10** | **2** |

samples from *Bosana*. This last variety also showed a remarkable biodiversity on olives and pastes (Table 1). It is noteworthy to highlight the presence of the two species *C. adriatica* and *C. molendiniolei* in the olive oils produced in Sardinia, as they were previously only identified in Central Italy (Molise and Umbria regions), Croatia (Cadez et al., 2012) and Tuscany (Mari et al., 2016). *C. dendronema* not appear to be previously isolated from olives or oil.

*Bacteria*. The sequencing of 16s rDNA of 19 strains isolated from olives, olive pastes and olive oils revealed that the isolates belonging manly to *Proteobacteria* phyla (5 genera, four of which belonged to *Enterobacteriaceae* family), followed by *Actinobacteria* (1 genus) and *Firmicutes* (2 genera) (Table 2). All isolates were identified at genus level, and only 9 strains were identified up to the level of species. As expected the microbiota biodiversity in olives was higher than oil; the latter having *Serratia* spp. as dominant genus, although this genus was present also in the olives with two species *Serratia ficaria* and *Serratia plymuthica*.

The culturable (cultivable) bacterial community of the olives of the three cultivars in terms of microbial species was quite different as shown in Table 2. *Bosana* olives contained the largest number of species when compared with the other two cultivars *Semidana* and *Nera di Gonnos*. *Raoultella terrigena* and *Kluyvera intermedia* species were found only in Nera di Gonnos while in Semidana the culturable (cultivable) community was dominated by *Pseudomonas* genus (Table 2). Bacteria isolates belonging to *Enterobacteriaceae* family were the main components of culturable community of olive oligotrophic environment, as found in the bacterial communities of the surface of others plants such as spinach, lettuce, and tomatoes (Leff and Fierer, 2013). Strains belonging to *Serratia* spp. were found in two out of three cultivars, and the strains identified up to level species belonged to *S. ficaria* and *S. plymuthica* species, which are phylogenetically distinct. In fact, *S. ficaria* is generally associated with the fig and fig wasp, even if biogenic amine-produce strains were isolated from meat (Grimont et al., 2005). *S. ficaria* is also an

**Table 2**
Bacteria Isolates divided *per* species and olive varieties.

| Bacteria species | N° isolates | Olive Varieties | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Bosana* | | | *Semidana* | | | *Nera di Gonnos* | | | *Nocellara del Belice* | | | | |
| Olive | Paste | Oil | Olive | Paste | Oil | Olive | Paste | Oil | Olive | Paste | Oil | | |
| *Serratia plymuthica* | 3 | 2 | – | – | – | – | – | 1 | – | – | – | – | – |
| *Serratia ficaria* | 2 | 2 | – | – | – | – | – | – | – | – | – | – | – |
| *Serratia* spp. | 4 | 1 | – | 3 | – | – | – | – | – | – | – | – | – |
| *Pseudomonas azotoformans* | 1 | – | – | – | – | – | – | 1 | – | – | – | – | – |
| *Pseudomonas fragi* | 1 | – | – | – | – | – | – | 1 | – | – | – | – | – |
| *Pseudomonas* spp | 2 | – | – | – | – | – | – | 1 | – | – | – | – | |
| *Raoultella terrigena* | 1 | – | – | – | 1 | – | – | – | – | – | – | – | |
| *Kluyvera intermedia* | 1 | – | – | – | 1 | – | – | – | – | – | – | – | – |
| *Staphylococcus* spp. | 1 | – | – | – | – | – | – | – | – | 1 | – | – | – |
| *Erwinia* spp | 1 | 1 | – | – | – | – | – | – | – | – | – | – | – |
| *Bacillus* spp | 1 | – | 1 | – | – | – | – | – | – | – | – | – | – |
| *Curtobacterium* spp | 1 | 1 | – | – | – | – | – | – | – | – | – | – | – |
| ***Total*** | **19** | **7** | **1** | **3** | **2** | **-** | **-** | **4** | **-** | **1** | **-** | **-** | **-** |

opportunistic pathogen responsible for intestinal colonization or serious infections such as septicaemia, gall bladder empyema in immunocompromised patients (Curiel et al., 2011; Gul et al., 2011). In contrast, *S. plymuthica* species are usually selected as biocontrol agent (Aisyah et al., 2016; Jankiewicz and Swiontek Brzezinska, 2015; Rybakova et al., 2016; Syafriani et al., 2016). Some of the isolates belong to *Erwinia* spp. Several strains of this species are endosymbionts of wild olive flies and are characterized by an intracellular and extracellular lifestyles (Estes et al., 2009), thus highlighting the important role of insects in affecting the composition of plant-associated communities (Pascazio et al., 2015).

There are several factors that could be influencing the structure and composition of olive microbial communities, as for instance, harvesting, handling, transport, and storage. Therefore it is difficult to unequivocally determine which specific factor or combination of factors was responsible for driving the divergence between bacterial communities on different olive cultivar (Leff and Fierer, 2013).

In our knowledge, this is the first study where bacterial were isolate from extra virgin olive oil. Microbial growth in extra-virgin olive oil is very unlikely because it does not contain sugars and nitrogenous compounds, and the water content is very low. All these conditions limit the growth of microorganisms (Alamprese, 2014). But their role in lipolysis, as described elsewhere, should not be underestimated.

As already observed for yeast, the presence of bacteria in the olive oil may be dangerous for commercial oil quality, due to the potential increase in free acidity, peroxide and spectrophotometric values and formation of sensory defects (Alamprese, 2014). The bacteria found in the oil belonged to *Serratia* spp. and *Staphylococcus* spp. genera. *Serratia* were also found in the olive fruits, therefore olives could be considered as reservoir of this bacterial species. The microbial communities of olive surface might be a source of new strains to use to reduce the pollutant produce during the olive processing. In fact a strain of *R. terrigena*, another species isolated from olive in this work, is able to breakdown the pollutants of olive washing water (Maza-Márquez et al., 2017), even if a case of neonatal infection was reported recently (Demiray et al., 2016), therefore caution could be taken when new isolates were selected from biological treatment of olive washing water and olive mill wastewater.

### 3.2. Enzymatic activities of isolates

Most of the microorganisms isolated in this work, have been previously isolated from other oleic ecosystems (Zullo et al., 2013). The positive or negative action of microorganisms on the oils depends on their enzymatic activities (Ciafardini and Zullo, 2002). Results for yeast and bacteria are summarized in Tables 3 and 4 respectively, which are consistent with data reported in literature for other geographic regions. Indeed, the enzymatic activities of yeasts found in olive oil microbiota (β-glucosidase β-glucanase, peroxidase, lipase and cellulose), are involved in the reduction of phenolic or other olive oil compounds (Romo-Sanchez et al., 2010). So, yeast and presumably bacteria, are also potentially able to produce changes in sensory olive oil profile because they are involved in the olive oil debittering process and phenolic compounds reduction during its storage (Zullo et al., 2013).

*Yeast*. As regards the β-glucosidase activity, all the isolates were able to show this activity at different magnitude. This activity is important because the enzyme β-glucosidase degrades the oleuropein, the main phenolic compound present in olives, in an eterosidic ester of the elenoic acid and in the 3-4-dihydroxyphenyl ethanol. The activity of these compounds is considered of great technical importance in view of their ability to turn the oil brown and give an intense bitter taste (Ciafardini and Zullo, 2002). However, only 11 isolates were able to grow on olive oils, probably due to the unfavorable environmental conditions, while 27 were able to survive to the paste.

The β-glucanase activity resulted positive in 28 isolates, where was more pronounced for species such as *C. molendiniolei*, *C. carnescens* and *A. pullulans*. The β-glucanase hydrolyzes secoiridoid glycosides, releasing antioxidant molecules (Iconomou et al., 2010).

**Table 3**
Yeast enzymatic activities.

| Yeasts species | N° isolates | Enzymes activity | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| β-glycosidase | | | | β-glucanase | | | Decarboxylase | | Catalase | | Lipase | | | | |
| 0 | 1 | 2 | 3 | 0 | 1 | 2 | (+) | (−) | (+) | (−) | (+) | (−) | | | |
| *Candida molendinolei* | 11 | – | – | 5 | 6 | 3 | | 8 | 1 | 10 | 6 | 5 | 2 | 9 | |
| *Candida adriatica* | 7 | – | – | 2 | 5 | 3 | 3 | 1 | 2 | 5 | 6 | 1 | 3 | 4 | |
| *Saccharomyces cerevisiae* | 6 | 1 | | 2 | 3 | 3 | 2 | 1 | – | 6 | 3 | 3 | – | 6 | |
| *Cryptoccoccus carnescens* | 5 | – | – | – | 5 | – | – | 5 | – | 5 | – | 5 | 5 | – | |
| *Aureobasidium pullulans* | 5 | – | – | – | 5 | – | – | 5 | 1 | 4 | – | 5 | 2 | 3 | |
| *Rhodotorula glutinis* | 3 | – | – | – | 3 | 2 | 1 | – | – | 3 | – | 3 | 2 | 1 | |
| *Candida wickerhami* | 3 | – | – | 3 | – | 3 | – | – | – | 3 | 3 | – | – | 3 | |
| *Pichia manshurica* | 2 | – | – | 2 | – | 2 | – | – | – | 2 | 2 | – | 2 | – | |
| *Nakazawaea anatomiae* | 2 | | | 2 | | 1 | | 1 | | 2 | 2 | | | 2 | |
| *Candida temnochilae* | 2 | – | – | 1 | 1 | 2 | – | – | 2 | – | 1 | 1 | 2 | – | |
| *Cryptococcus magnus* | 1 | – | – | – | 1 | – | – | 1 | – | 1 | – | 1 | 1 | – | |
| *Pichia mexicana* | 1 | – | – | – | 1 | 1 | – | – | 1 | – | – | 1 | 1 | – | |
| *Rhodosporium babjevae* | 1 | – | – | – | 1 | 1 | – | – | – | 1 | – | 1 | 1 | – | |
| *Candida diddensiae* | 1 | – | – | – | 1 | 1 | – | – | – | 1 | – | 1 | 1 | – | |
| *Candida guilliermondii* | 1 | | | | 1 | 1 | | | | 1 | 1 | | 1 | | |
| *Cryptococcus oeirensis* | 1 | – | – | – | 1 | – | – | 1 | – | 1 | – | 1 | – | 1 | |
| *Cryptococcus victoriae* | 1 | – | – | – | 1 | – | – | 1 | – | 1 | – | 1 | – | 1 | |
| *Candida dendronema* | 1 | – | – | – | 1 | 1 | 1 | – | – | 1 | 1 | 0 | 1 | – | |
| *Pirula Salina* | 1 | – | – | – | 1 | – | 1 | – | – | 1 | – | 1 | 1 | – | |
| *Pichia nakazawaea* | 1 | – | – | 1 | – | 1 | – | – | – | 1 | 1 | – | 1 | – | |
| *Candida* sp *CLIB 1308* | 1 | – | – | 1 | – | – | – | 1 | 1 | – | 1 | – | – | 1 | |
| *Rhodororula* spp | 1 | – | – | | 1 | 1 | | | – | 1 | | – | 1 | | |
| *Others* | 6 | | | 1 | 5 | 3 | | 3 | 1 | 5 | 3 | 3 | 3 | 3 | |
| **Total** | **64** | **1** | **-** | **20** | **43** | **29** | **7** | **28** | **9** | **55** | **30** | **34** | **30** | **34** | |

**Table 4**
Bacteria enzymatic activities.

| Bacteria | N° isolates | Enzymes activity | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| β-glycosidase | | | | β-glucanase | | | Decarboxylase | | Catalase | | Lipase | | | |
| 0 | 1 | 2 | 3 | 0 | 1 | 2 | (+) | (−) | (+) | (−) | (+) | (−) | | |
| *Serratia plymuthica* | 3 | – | – | – | 3 | 1 | 2 | – | 3 | – | 3 | – | 3 | – |
| *Serratia ficaria* | 2 | – | – | – | 2 | – | 2 | – | 2 | – | 2 | – | 2 | – |
| *Serratia* spp. | 4 | – | – | – | 4 | 3 | 1 | – | 4 | – | 4 | – | 4 | – |
| *Pseudomonas azotoformans* | 1 | – | – | – | 1 | 1 | – | – | 1 | – | – | 1 | 1 | – |
| *Pseudomonas fragi* | 1 | – | – | – | 1 | 1 | – | – | 1 | – | 1 | – | 1 | – |
| *Pseudomonas* spp | 2 | – | – | – | 2 | 2 | – | – | 2 | – | 1 | 1 | 1 | 1 |
| *Raoultella terrigena* | 1 | – | – | – | 1 | – | – | 1 | – | 1 | – | 1 | 1 | – |
| *Kluyvera intermedia* | 1 | – | – | – | 1 | | 1 | – | – | 1 | – | 1 | 1 | – |
| *Staphylococcus* spp. | 1 | – | – | 1 | – | 1 | – | – | 1 | – | 1 | – | 1 | – |
| *Erwinia* spp | 1 | – | – | – | 1 | – | 1 | – | 1 | – | 1 | – | 1 | – |
| *Bacillus* spp | 1 | – | – | – | 1 | 1 | – | – | – | 1 | – | 1 | 1 | – |
| *Curtobacterium* spp | 1 | – | – | – | 1 | – | – | 2 | – | 1 | – | 1 | – | 1 |
| ***Total*** | **19** | | | **1** | **18** | **9** | **7** | **3** | **15** | **4** | **13** | **6** | **17** | **2** |

The concentration of secoiridoid glycosides in olive oil depends on the variety of olive and state of ripeness of the fruit (Ciafardini and Zullo, 2002b). The secoiridoids, with important nutritional properties (Visioli et al., 1995), are the main antioxidants present in olive oil.

As regards to the dexarboxylase activity, only 9 isolates over 64 showed this activity. Yeast were analyzed in Petri dish plates containing the single amino acids first, the and then analyzed with the AAs mix (see section 2.4). It is important to underline that only yeast that were able to show a surrounding halo in one of the single aa test were considered positive. The decarboxylation is, on the opposite, considered a negative activity, because leads to the production of biogenic amines able to trigger allergic and inflammatory reactions in humans. Since different species of yeast are responsible for the synthesis of biogenic amines, this enzymatic activity should be carefully considered (Bevilacqua et al., 2013).

A number of 30 above 64 isolates, showed peroxidase activity, with the exception of those belonging to the genus *Cryptococcus* and species *A. pullulans* and *R. glutinis*. This activity adversely affects both the salubrity and organoleptic qualities of olive oils, as they degrade the phenolic compounds and polyphenols. These compounds possess antioxidant properties, able to prevent rancidity (Angerosa et al., 2000), they also possess anti-inflammatory, anti-allergic and anti-bacterial properties and contribute to the fruity, spicy and bitter taste, which are important distinctive characters in extra virgin olive oils.

Last activity tested was the lipolytic activity. Olives are fruits with high fat concentrations and the presence of lipolytic yeasts could modify the nutritional composition of the final product (Arroyo-López et al., 2008). In fact, lipase-producing yeasts can impair product quality due to the increase of both the diglyceride and acidity levels through hydrolysis of triacylglycerols (Cardenas et al., 2001; Zullo and Ciafardini, 2008). 30 out of 64 isolated yeast showed this activity. Among those not showing this activity are *S. cerevisiae* and *C. wickerhami* species (Table 3). Finally, 6 isolates (*S. cerevisiae* GGR6, *A. pullulans* SOV8, *C. molidiniolei* B2GR2, *S. cerevisiae* SGR1 and SGR2, and *P. mexicana* NOL1) showed only positive enzymatic activities and they could be potential candidate for technological utilization.

*Bacteria*. Almost all the isolates showed β-glucosidase, decarboxylase and lipase activities, but not β-glucanase and catalase (Table 4). Particularly, all 19 isolates showed β-glucosidase activity, while decarboxylase activity was showed in 15, β-glucanase in 10, catalase in 13 and lipase in 17. Only one of the bacterial strain, isolate from olives, the *Curtobacterium* spp, showed positive activities (Table 4). Recently it has been found that this genus plays a dominant role in the functional breakdown of dead organic material in leaf litter communities, due to its ability to degrade carbohydrates, (Chase et al., 2016).

### *3.3. Biofilm and mat formation*

The ability of some yeast to adhere to plastic and form biofilm, opens new discussion about the possibility that these yeast (that belong to emergent pathogens), could be dangerous for human health. In addition, recent observations of the presence of some emerging yeast pathogens in the oils (Zullo et al., 2010; Ciafardini et al., 2013) determined the further analyses carried in the present work on the ability of the isolates to adhere to plastic and form a cellular mat. Within 64 yeast isolated, 23 were able to adhere to the plastic and to form biofilm (Table 5). Among them, the oil-born yeast *C. moliindiniolei, C. adriatica, C, wickerhami, C. diddensiae and S. cerevisiae*, but also some emergent pathogens like *C. gulliermondii* and *C. car-*

**Table 5**
Biofilm forming yeast.

| Biofilm formation | | | |
|---|---|---|---|
| Yeasts species | N° isolates | Adhesion to plastic | |
| (+) | (−) | | |
| *Candida molendinolei* | 11 | 6 | 5 |
| *Candida adriatica* | 7 | 4 | 3 |
| *Saccharomyces cerevisiae* | 6 | 2 | 4 |
| *Cryptococcus carnescens* | 5 | 2 | 3 |
| *Aureobasidium pullulans* | 5 | – | 5 |
| *Rhodotorula glutinis* | 3 | 1 | 2 |
| *Candida wickerhami* | 3 | 3 | – |
| *Pichia manshurica* | 2 | – | 2 |
| *Nakazawaea anatomiae* | 2 | 1 | 1 |
| *Candida temnochilae* | 2 | 1 | 1 |
| *Cryptococcus magnus* | 1 | – | 1 |
| *Pichia Mexicana* | 1 | 1 | – |
| *Rhodosporium babjevae* | 1 | – | 1 |
| *Candida diddensiae* | 1 | – | 1 |
| *Candida guilliermondii* | 1 | 1 | – |
| *Cryptococcus oeirensis* | 1 | – | 1 |
| *Cryptococcus victoriae* | 1 | – | 1 |
| *Candida dendronema* | 1 | 1 | – |
| *Pirula Salina* | 1 | – | 1 |
| *Pichia nakazawae* | 1 | 1 | – |
| *Candida* sp *CLIB 1308* | 1 | – | 1 |
| *Rhodororula* spp | 1 | 1 | – |
| *Others* | 6 | – | 6 |
| **Total** | **64** | **25** | **39** |

*nescens* were able to form a biofilm. Moreover, a yeast found for first time in the oleic system, *C. dendronema*, was able to form a biofilm. This high number of dimorphic yeast in the oleic system can be critical when consideraing that the dimorphism is associated with pathogenicity, even though these yeast are very sensitive to oil components and so they tend to disappear from the final oil (Zullo et al., 2010).

The biofilm forming candidates were also subjected to mat formation, that is the ability of yeast to have sliding motility in order to forage for nutrients. Not all the yeast able to form a biofilm were also able to produce a mat on semisolid medium. Most of the yeast mats are summarized in Fig. 1. This activity, as underlined above, is a feature that most of the biofilm forming yeast have, and it is correlate to the abil-

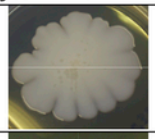
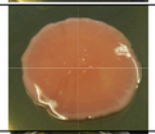
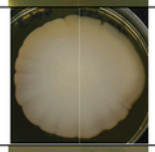
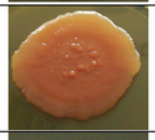
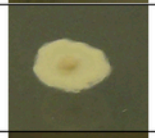
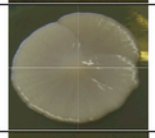
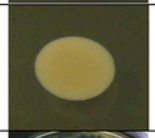
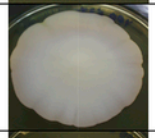
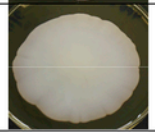
| ***Origin*** | ***Species*** | ***Mat formation after 21 d*** |
|---|---|---|
| Semidana Paste 2 | *Saccharomyces cerevisiae* |  |
| Bosana1 Olives 7 | *Rhodotorula glutinis* |  |
| **Nera of Gonnos Oil 3** | ***Candida temnochile*** |  |
| Nera of Gonnos Paste 3 | *Candida molidini-olei* |  |
| Semidana Olives 12 | *Rhodotorula spp* |  |
| **Semidana Oil 3** | ***Candida adriatica*** |  |
| **Semidana Oil 1** | ***Candida adriatica*** |  |
| **Semidana Oil 2** | ***Candida adriatica*** |  |
| Nocellara Olives 4 | *Cryptococcus carnescens* |  |
| **Nocellara Oil 3** | ***Candida dendronema*** |  |
| **Nera of Gonnos Oil** 1 | ***Pichia nakazawaea*** |  |

**Fig. 1.** Mat formation of the most representative biofilm forming yeast, (in bold dimorphic yeast isolated from olive oils).

**Table 6**
Probiotic test for the identified yeast.

| Yeasts species | N° isolates | YPD pH 2.5 (2 h) | | YPD + bile salt 1.5% (2 h) | |
|---|---|---|---|---|---|
| | (+) | (−) | (+) | (−) | |
| *Candida molendinolei* | 14 | 12 | 2 | 12 | 2 |
| *Candida adriatica* | 7 | 3 | 4 | 5 | 2 |
| *Saccharomyces cerevisiae* | 6 | 6 | – | 6 | – |
| *Cryptoccoccus carnescens* | 5 | – | 5 | 5 | – |
| *Aureobasidium pullulans* | 5 | 4 | 1 | 4 | 1 |
| *Rhodotorula glutinis* | 3 | – | 3 | 2 | 1 |
| *Candida wickerhami* | 3 | 3 | – | 3 | – |
| *Pichia manshurica* | 2 | 2 | – | 2 | – |
| *Nakazawaea anatomiae* | 2 | 2 | – | 2 | – |
| *Cryptococcus magnus* | 1 | – | 1 | 1 | – |
| *Pichia mexicana* | 2 | 2 | – | 2 | – |
| *Rhodosporium babjevae* | 1 | 1 | – | 1 | – |
| *Candida diddensiae* | 1 | 1 | – | 1 | – |
| *Candida guilliermondii* | 1 | 1 | – | 1 | – |
| *Cryptococcus oeirensis* | 1 | 1 | – | 1 | – |
| *Cryptococcus victoriae* | 1 | – | 1 | 1 | – |
| *Candida dendronema* | 2 | 2 | – | 2 | – |
| *Pirula Salina* | 1 | – | 1 | 1 | – |
| *Pichia nakazawaea* | 1 | 1 | – | 1 | – |
| *Debaryomyces hansenii* | 1 | 1 | – | 1 | – |
| *Fidobasidium magnum* | 1 | – | 1 | 1 | – |
| *Others* | 3 | 2 | 2 | 4 | – |
| **Total** | **64** | **43** | **21** | **58** | **6** |

**Table 7**
Probiotic test for the identified bacteria.

| Bacteria | N° isolates | YPD pH 2.5 (2 h) | | YPD + bile salt 1.5% (2 h) | |
|---|---|---|---|---|---|
| | (+) | (−) | (+) | (−) | |
| *Serratia plymuthica* | 3 | 1 | 2 | 3 | – |
| *Serratia ficaria* | 2 | – | 2 | 2 | – |
| *Serratia* spp. | 4 | – | 4 | 4 | – |
| *Pseudomonas azotoformans* | 1 | – | 1 | 1 | – |
| *Pseudomonas fragi* | 1 | 1 | – | 1 | – |
| *Pseudomonas* spp | 2 | 1 | 1 | 2 | – |
| *Raoultella terrigena* | 1 | 1 | – | 1 | – |
| *Kluyvera intermedia* | 1 | – | 1 | 1 | – |
| *Staphylococcus* spp. | 1 | – | 1 | 1 | – |
| *Erwinia* spp | 1 | – | 1 | 1 | – |
| *Bacillus* spp | 1 | 1 | – | – | 1 |
| *Curtobacterium* spp | 1 | 1 | – | 1 | – |
| ***Total*** | **19** | **6** | **13** | **18** | **1** |

ity to survive to starving for nutrients. Adherence to plastic surface and mat formation are frequently associated and can be considered as indexes of pathogenicity (Kalai Chelvam et al., 2014). So the high number of yeast able to form a biofilm (25 over 64) and among them the high number of yeast able to form a mat (22 over 25) could be related to the selection of biofilm forming yeast of the oleic system. It is known, indeed, that yeast survive in this environment only because the presence of water drops (Ciafardini and Zullo, 2002), therefore the strong selection of the oleic environment could have selected this large number of dimorphic yeast able to form a cellular mat. Considering the high number of yeast able to form a biofilm, the possibility that these yeast can also adhere and survive on the walls of the oil reservoirs cannot be discarded. Finally, biofilm and mat formation were also tested on bacteria isolates, but none of the tested strains resulted positive to the adherence to plastic nor mat formation (results not shown).

### *3.4. Probiotic tests*

Acidic and bile salt resistance tests showed that the yeast and bacteria isolated from the analyzed oleic systems could be potentially used as probiotics, and their presence in the oils at low concentrations can be seen positively. Among 64 yeast isolates 40 were resistant to pH 2.5 and 55 isolates to 1.5% bile salt, while among 19 bacteria isolated, 6 were resistant to pH 2.5 and 18 strains to 1.5% bile salt (Tables 6 and 7). Considering the probiotic potentiality of these isolates, their presence in the olive oils, or their utilization in other food matrices, could be considered positively, especially if not characterized by negative enzymatic or potential pathogen activities. Particularly, isolates GGR6 and SGR1 belonging to *S. cerevisiae*, SOV8 belonging to *Aureobasidium pullulans* and B1OV13 belonging to *Curtobacterium* spp resulted biofilm, decarboxylase, catalase and lipase negative, but in the meantime are β-glucosidase, β-glucanase and positive to the probiotic tests. To our knowledge there is no other study that emphasizes the presence of potential probiotic microorganisms in olive oils.

## 4. Conclusions

This work highlights that in 4 varieties of olives, olive pastes and oils sampled in Sardinia, a high microbial biodiversity has been observed, and it also confirms that some yeast and bacteria species can be generally considered distinctive of oleic ecosystems (Ciafardini et al., 2002, 2017). Most of the yeast genus found in this work are, indeed, typical of the oleic ecosystem such as *Candida, Pichia, Saccharomyces* (Romo-Sanchez et al., 2010), while other genera are less common, such as *Cryptococcus*. Among bacteria we found that mostly of the isolates are typical of the soil ecosystem, especially *Serratia* spp and *Pseudomonas* spp. Between the four analyzed olive varieties, the *Bosana* oil was the one with the highest microbial biodiversity. In general, an effect of selection of the number of isolates in the passage from olives to malaxation to oils has been observed. Some isolates present in the production process initial stages are not present in the subsequent stage, others are present only in the later stages. This phenomenon has been observed for all cultivars under examination, and is it presumably due to the selective effect during malaxation. Some yeast present in the olives at malaxation stage, are reduced and/or eliminated by increased temperature typical of this step or by the presence of molecules with antimicrobial activities such as polyphenols. On the contrary, other yeast species are found only in the oil, maybe due to the selection and enrichment phenomena inside of each single mill. Considering what recently reported by Vichi et al. (2011), the metabolic activities of olive microbiota during the extraction process could be a critical point for virgin olive oil, and their influences could be greater than those exerted by malaxation time and temperature. In these terms, and considering what we found in this study, yeast and bacteria species contaminating the mill might be also a problem for the sensorial quality of virgin olive oils. In addition, the presence of biofilm forming yeast in the oil, which could be considered as emergent pathogens, has to be considered and further investigated. Finally, yeast and bacteria isolates presented a higher resistance to bile acid than opening to further studies aimed to determine the health and safety of these strains and their role as po-

tential probiotic microorganisms to be used in olive oils or other food matrices.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2017.09.004.

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