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Effect of lipolytic activity of *Candida adriatica*, *Candida diddensiae* and *Yamadazyma terventina* on the acidity of extra-virgin olive oil with a different polyphenol and water content

G. Ciafardini, B.A. Zullo*

Department of Agricultural, Environmental and Food Sciences, University of Molise, Via De Sanctis, I-86100, Campobasso, Italy

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ABSTRACT

Previous microbiological research demonstrated the presence of a rich micro-flora composed mainly of yeasts in the suspended fraction of freshly produced olive oil. Some of the yeasts are considered harmful as they can damage the quality of the olive oil through the hydrolysis of the triacylglycerols. Present research has demonstrated that the lipolytic activity of some lipase-producer strains belonging to a yeast species called *Candida adriatica*, *Candida diddensiae* and *Yamadazyma terventina* can be modulated by the water and the polyphenol content of olive oil. Laboratory tests highlighted a substantial increase in free fatty acid in the inoculated olive oil characterized by high water content and low polyphenol concentration. The acidity of the olive oil samples containing 0.06% and 0.31% of water increased significantly by 33% in the lipase-producer yeast strains tested during a period of 2 weeks of incubation at 30 °C. All other yeasts showed strong lipolytic activity in the presence of 1.31% of water – the only exception to this was the *C. adriatica* 1985 strain. The phenolic compounds typical of olive oil represent another important factor able to condition the viability and the lipolytic activity of the lipase-producer yeasts. From the tests performed on the olive oil characterized by an increasing content of total polyphenols equal to 84, 150 and 510 mg per kg of oil, the percentage of the lipase-producer yeasts able to hydrolyse the triacylglycerols was respectively 100%, 67% and 11%.

1. Introduction

Olive oil represents the main vegetable oil source used in the Mediterranean area and its use is now spreading rapidly to other areas such as the U.S. and Australia. Extra virgin olive oil is obtained from the fruits of the olive tree using an exclusively mechanical process and is one of the oldest edible oils that can be consumed without any refining process. The quality of olive oil based on chemical parameters includes the presence of free fatty acids. In line with European Community regulation 299/2013 (EC, 2013), less than 0.8% of total free fatty acids, expressed as oleic acid, is required in the production of extra-virgin olive oil. A hydrolase family of enzymes, the lipase, are involved in the production of the free fatty acids in olive oil. The lipase (EC-3.1.1.3) catalyses the hydrolysis of water-insoluble ester such as triacylglycerols. These enzymes possess the unique feature of acting as an interface between the aqueous and non-aqueous (i.e. olive oil) phases and this feature distinguishes them from esterases (Macre and Hammond, 1985). The lipases hydrolyse the ester-carboxylate bonds of acylglycerols producing free fatty acids, partial acylglycerols and glycerol. The lipases originating from the fruits are the main cause of the acidity of newly produced olive oil coming from olives damaged before processing (Frega et al., 1999). However, recent microbiological research has proved that freshly produced olive oil is contaminated by microorganisms capable of conditioning the physical-chemical and organoleptic characteristics of the oil through the production of enzymes (Ciafardini and Zullo, 2002a, b; Zullo et al., 2013). The microorganisms found in olive oil are mainly those from yeasts originating from the olives' phyllosphera. During the processing of the olives in the mill they migrate into the oil where they reproduce in a selective way depending on the chemical composition of the oil and go on to constitute the typical microbiota of each oil (Ciafardini et al., 2004). Some of these microorganisms were found to be beneficial as they produce a β-glucosidase and esterase capable of increasing the quality of the more bitter olive oil through the hydrolysis of the glucoside known as oleuropein into simpler and no

* Corresponding author. Tel.: +39 874 404689; fax: +39 874 404855.
E-mail address: biagi.zullo@unimol.it (B.A. Zullo).

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longer bitter compounds such as hydroxytyrosol and elenolic acid characterized also by a high antioxidant activity. Nevertheless, other forms of yeasts that increase acidity through the production of lipase during olive oil storage can compromise oil quality. Lipase activity has been noticed in many oil-born yeast forms of *Candida wickerhamii* and *Candida parapsilosis* species (Zullo and Ciafardini, 2008b). Other research, carried out on the distribution of dimorphic yeast species in commercial extra-virgin olive oil, showed both the presence of lipase-producer (Lip^+) and Lip^- strains of *Candida diddensiae* in different olive oil samples (Zullo et al., 2010, 2013). However, olive oil is a heterogeneous habitat where yeasts with biochemical and genetic characteristics not identical to known yeast species also live. In fact, Cadez et al. (2012, 2013) described new yeast species called *Candida adriatica*, *Candida molendinolei*, *Ogataea kolombanensis, Ogataea histrianica* and *Ogataea deakii.* These species have been isolated from olive oil and its by-product. Ciafardini et al. (2013) report a new yeast species from Italian olive oil, namely *Yamadazyma terventina*. Some of these new species such as *C. adriatica* and *Y. terventina* as well as *C. diddensiae*, were found frequently in extra-virgin olive oils of the Leccino variety produced in central Italy, but the role that they play during the storage of the oil is not well-known. In fact, to date, it is not possible to know if their enzymatic activity is able to improve or not the chemical and sensory characteristics of freshly produced olive oil during its storage. One of the possible negative effects of some yeasts on olive oil quality refers to the increase in acidity promoted by the lipase of microbial origin active on the triacylglycerol. However, at the present state of knowledge, it is still not completely clear whether the enzymatic degradation of the olive oil triacylglycerols is due mainly to the lipases from the olive fruits crushed in the mill or by those of microbial origin produced by the oil microbiota. Previous micro-biological research indicated that certain Lip^+ strains of *Williopsis californica* and *Saccharomyces cerevisiae*, inoculated in the same oil from which they were isolated, increased the extra virgin olive oil acidity respectively from 0.62% to 1.50% and 1.62% during a two-week incubation period. This exceeded the limit of 0.8% established by current regulations for this commercial category of olive oil (Ciafardini et al., 2006a,b; EC, 2003, n. L. 295/57). Given that the lipase can have a negative effect on the quality of olive oil during its storage, a study has been conducted on the lipolytic activity of Lip^+ strains of a yeast commonly found in Italian olive oil, namely *C. diddensiae* and other two new yeast species recently discovered in olive oil, namely *C. adriatica* and *Y. terventina*. Both studies were carried out on olive oil characterized by a different polyphenols and water content.

2. Material and methods

2.1. Enzymatic activity and yeast growth behaviour

The yeast strains used in the trials had been previously isolated from extra-virgin olive oil and classed through physiological and genetic analysis as *C. adriatica* (Cadez et al., 2012), *C. diddensiae* (Zullo et al., 2010) and *Yamadazima terventina* (Ciafardini et al., 2013) species. The yeasts were grown for 3 days at 30 °C in Petri dishes with MYGP agar medium containing: 3 g yeast extract (Biolife, Milan, Italy), 3 g malt extract (BBL, Cockeysville, MD, USA), 5 g beef extract powder (BBL), 10 g D-glucose (Merck, Darmstadt, Germany), 1000 mL distilled water, pH 7, as described by Kurtzman and Fell (1998). All the yeast cultures were further characterised by taking into consideration dimorphic growth behaviour and some important enzymatic activity involved in the hydrolysis of the synthetic esters as well as the olive oil triacylglycerol compounds. The chromogenic characteristics of the yeast colonies were evaluated by cultivating the cells on the MYGP agar medium for 5 day at 30 °C. At the end of incubation a series of masters were set up with the yeast strains using the CHROMagar Candida medium (BBL 4354093, Madison, USA). Transfer of the colonies on CHROMagar Candida medium was carried out using small wooden sticks sterilised in an autoclave at 120 °C for 30 min. Both the colony morphology and their colour were evaluated after 7 days incubation at 30 °C as described by Tornai-Lehoczki et al. (2003). The influence of nutritional stress condition on the growth behaviour of the above-reported dimorphic yeasts was evaluated by considering the appearance of the pseudohyphae during the growth phase in a poor medium represented by the corn-meal agar medium (CMA-BBL, USA), containing 1% (v/v) of polysorbate 80 (tween 80) as described by Kelly and Funigeillo (1959). The microscopic observations were carried out after 9 days of incubation at 25 °C using an Olympus mod. BX50F4 (Olympus Optical Co., Japan) light microscopy fitted with a camera. All the experiments were performed with three repetitions. The lipase tests were conducted both with synthetic and natural substrates. The trials with the synthetic chromogen substrates were accomplished by transferring 30 mg of each yeast culture biomass into the test tubes containing 10 mL of substrate. The synthetic chromogen substrates used were *p*-nitrophenylbutirate, *p*-nitrophenylcaprate, *p*-nitrophenylcaproate and *p*-nitrophenylpalmitate (Sigma–Aldrich Co, USA). The trials were carried out as described by Jin-Kyn et al. (2005) with some modifications. In detail, each substrate described above was dissolved in 400 μL of acetonitrile, then 40 mL of 2-N-morpholino ethanesulfonic acid sodium salt (MES) hydrate (Sigma–Aldrich Co, USA), 50 mM pH 6, was added to reach a final substrate concentration of 3 mM. Each solution was mixed briefly using a magnetic stirrer. It was then transferred separately into the test tubes. Four repetitions and one negative control without the yeast biomass were accomplished. The test tubes were swirled for 1 min with a vortex and then incubated in agitation (50 rpm) at 30 °C. After 150 min of incubation the reaction was stopped without interfering with the reactants, by the addition of 50 μL of a 40% (w/v) NaOH solution. The mixture was then centrifuged (Universal 32 centrifuge, Hettich, Tuttlinger, Germany) at 10,000 × *g* for 5 min and the supernatants were analysed with a model 6300 Jenway spectrophotometer (U.K.) at 410 nm, zeroing the instrument with the negative control without the yeasts' biomass. The lipase activity in the presence of natural substrates was evaluated in sterile test tubes containing 10 mL of filter-sterilized extra-virgin olive oil using a white 47 mm sterile filter with 0.45 μm of porosity (Millipore Co, MA 01821) and 30 mg of the above yeast strains' biomass. The extra-virgin olive oil, produced by the Leccino variety, was characterized by the following analytical indices: free fatty acids, 0.60%; peroxide values, 8.7 mEq O_2 per kg oil; bitterness index 0.38; total polyphenols, 84 mg per kg oil and 0.31% of water content. Four repetitions and one negative control without yeast were also carried out for each strain. The test tubes were swirled 3 min with a vortex and then incubated 40 days in a dark place at 30 °C. At the end of the incubation the test tubes were centrifuged at 10,000 × *g* for 10 min and the oil was used for the chemical analysis of the acidity and fatty acid profiles. The acidity and the peroxide values were assessed according to the European Community regulation 299/2013 (EC, 2013). The profile of the free fatty acids of the same olive oil samples incubated for 40 days in the presence of the different yeast strains already described was made according to the European Community1429/92 Regulation (EC, 1992). The analysis of fatty acid methyl esters was performed using a Fisons HRGC (Mega 2 series) with a flame-ionisation detector and equipped with a WCOT fused-silica capillary column, FFAP-CB coating, film thickness 0.30 μm, 25 m in length × 0.32 mm i.d. from Chrompack (Middleburg, The Netherlands). The total polyphenols were isolated three times by extraction of a solution of oil in hexane with a water-methanol mixture (40:60, v/v). The