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Short communication

Taggiasca extra virgin olive oil colonization by yeasts during the extraction process

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ABSTRACT

The opalescent appearance of the newly produced olive oil is due to the presence of solid particles and microdrops of vegetation water in which the microorganisms from the olives' carposphere are trapped. Present research has demonstrated that the microbiota of the fresh extracted olive oil, produced in the mills, is mainly composed of yeasts and to a lesser extent of molds. The close link between the composition of the microbiota of the olives' carposphere undergoing to processing, and that of the microbiota of the newly produced olive oil, concerns only the yeasts and molds, given that the bacterial component is by and large destroyed mainly in the kneaded paste during the malaxation process. Six physiologically homogenous yeast groups were highlighted in the wash water, kneaded paste and newly produced olive oil from the Taggiasca variety which had been collected in mills located in the Liguria region. The more predominant yeasts of each group belonged to a single species called respectively: *Kluyveromyces marxianus*, *Candida oleophila*, *Candida diddensiae*, *Candida norvegica*, *Wickerhamomyces anomalus* and *Debaryomyces hansenii*. Apart from *K. marxianus*, which was found only in the wash water, all the other species were found in the wash water and in the kneaded paste as well as in the newly produced olive oil, while in the six-month stored olive oil, was found only one physiologically homogeneous group of yeast represented by the *W. anomalus* specie. These findings in according to our previous studies carried out on other types of mono varietal olive oils, confirms that the habitat of the Taggiascas' extra virgin olive oil, had a strong selective pressure on the yeast biota, allowing only to a few member of yeast species, contaminating the fresh product, to survive and reproduce in it during storage.
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1. Introduction

Olive oil is a basic component of the Mediterranean diet known for its high nutritional value and sensory characteristics. The habitat of extra virgin olive oil, unlike more common foods, was for many years considered unsuitable for microorganisms. However, recent microbiological research has proved that freshly produced olive oil is contaminated by microorganisms mainly composed of yeasts, capable of conditioning the physic-chemical and organoleptic characteristics of the oil through the production of enzymes. Some yeasts are considered useful as they improve the organoleptic characteristics of the oil during the preservation, whereas others are considered harmful as they can damage the quality of the oil through the hydrolysis of the triglicerides (Ciafardini and Zullo, 2002, 2015; Zullo and Ciafardini, 2008; Zullo et al., 2013). However, our current knowledge of the dynamics of the distribution of the olives' carposphere microorganisms in the different substrates produced in the mills during the fruits processing is still very limited (Romo-Sànchez et al., 2010; Guerrini et al., 2015). However, the data on the survival of these microorganisms, contaminating the newly produced olive oil during its storage are absent. In fact, it is not known if during the olive processing, some contaminant microorganisms are destroyed mainly in the products of the mills or if they are devitalised in the olive oil during its storage. Furthermore, it is unclear whether all or only some of the yeasts present in the newly produced olive oil are able to produce the future extra virgin olive oil microbiota. The aim of this work was to study the distribution of the fruits' carposphere microorganisms in the products of the mills during the olives' processing, as well as the microbiota composition of both the newly produced and the stored olive oil.

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2. Materials and methods

2.1. Sampling through olive oil extraction process

The trials were accomplished using olives, kneaded paste and olive oil produced in three olive mills located in the north western region of Liguria (Italy) in the Genoa (GE), Imperia (IM) and Savona (SV) areas, during the olive oil production of 2014. The Taggiasca olive, which is a typical variety of this geographical area, was harvested at the beginning of its period of maturation when an analysis of the fruits, carried out by following the procedures previously reported, indicated a reduction in time of the concentration of total polyphenols present in the oily fraction of the fruits (Ciafardini and Zullo, 2014; Zullo et al., 2014). The fruits were processed in ordinary conditions in three commercial three phase continuing oil mills (Pieralisi, Iesi, Italy) equipped with a fruit separator, wash water system, olive grinding cutter crusher, kneading equipment, low speed centrifuge (decanter) and a vertical centrifuge separator. The microorganisms were studied by analysing the wash water, the kneaded paste and the newly produced olive oil of single masses of olives during their phases of transformation. In detail, in each mill the same quantity of the Taggiasca variety of olive equal to 700 Kg, was processed within 12 h of the harvest. After having had all the leaves and other materials removed, the olives were washed by using circa 400 Kg of fresh tap water batches. The fruits were crushed at 2000 rpm with a grinder and the paste obtained underwent malaxation for 20 min at 25 °C and was then enriched with tap water. A double centrifugation followed to separate the oil from the other components of the fruit. Six samples of wash water, kneaded paste and olive oil respectively were taken in sterile condition during the process of oil extraction using 1L sterile plastic containers. Half of the samples were used immediately for microbiological analysis while the remaining part was stored at −20 °C and used for the routine chemical analysis of the oil in accordance with the European Community Regulation 640/2008 (EC, 2008).

2.2. Microbiological analysis

Total bacteria count was evaluated on Standard Plate Count Agar (Oxoid, Basingstoke, Hampshire, United Kingdom). The yeasts and molds were detected using 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). This medium was enriched with sodium propionate (2 g/L) and tetracycline (20 mg/L) in order to inhibit growth of moulds and bacteria respectively. The wash water samples, containing the carposphere microorganisms of the olives, were used directly for the decimal dilution preparation using sterile physiological solution with 0.9% (w/v) NaCl. The kneaded paste and the olive oil samples were used for decimal dilution preparation after the elimination of the oily fraction that impedes the adhesion of the cells on the surface of the inoculated solid medium. In detail, 10 g of kneaded paste was suspended in 20 mL of sterile distillate water. The suspension, after a brief agitation, was micro-filtered through sterile nitrocellulose filters with a porosity of 0.45 μm (Ministart NML-Sartorius, Göttingen, Germany). The nitrocellulose filter of each sample was then transferred into a 25 mL sterile beaker where they were homogenized with a Turrax mod. T25 homogenizer (IKA, Milan, Italy), in the presence of sterile physiological solution. Finally the initial weight of each sample was reconstitute through the addition of sterile physiological solution and finally used for the 10-fold serial dilution preparation as described before. The olive oil samples were pretreated in the same manner as the paste samples with the difference that in this case, the oil samples were micro-filtered directly without the addition of the sterile distillate water. 200 μL of the decimal dilution was plated onto the above specific growth media for colony counting in triplicate, using the spread plating technique. The bacteria and the yeasts colony were counted respectively after 72 h and 5 days incubation at 30 °C; whereas the molds were evaluated after 7 days incubation at 28 °C. The yeast colony forming units (CFU), grown on the MYGP agar medium, after counting, were isolated and used in the tests described below.

2.3. Evaluation of the yeast biodiversity

The biodiversity between the different yeast isolates found in the wash water, in the kneaded paste and in the newly extracted olive oil as well as in a six-months stored olive oil, was studied through the screening of a high number of colonies carried out on the basis of specific chromogenic markers and their ability to metabolize sugars. The identifiable characteristics of each chromogenic group had to do with the appearance in the colonies of the different colors as well as their distribution within each colony of the master. All the yeast colonies of different randomly selected Petri dishes, obtained by using the above microbiological analysis, were used for the preparation of masters in the Petri dish containing the CHROMagar Candida medium (BBL, cod. 4354093, Madison, USA). In detail, the yeasts colonies coming from samples of wash water, kneaded paste and olive oil, were used to set up a series of masters containing 50 colonies each one. Altogether we tested about 1800 yeasts using 360–500 isolated from each sample according to their initial concentration as evaluated using the previous microbiological analysis. The colony morphology and their color were evaluated after 7 days of incubation at 30 °C as reported by Tornai-Lehoczki et al. (2003). All yeasts tested in the Petri dish with CHROMagar Candida medium were grouped into six homogeneous chromogenic groups of colonies with the following characteristics: A: uniform green, B: uniform white, C: fire red centre and white exterior, D: heavenly centre and white exterior, E: uniform light brown, F: light brown centre and white exterior. Within each chromogenic group, for each sample of wash water, kneaded paste and olive oil, 50 yeast colonies were chosen at random and were first reproduced in MYGP broth for 48 h at 30 °C and were then tested for their ability to metabolize sugars using the Integral System Yeasts Plus (Liofilchem cod. 71822, Roseto d. Abruzzi, TE, Italy), following the instructions provided by the manufacturer. The results obtained from each yeast culture were recorded, then, taking in consideration the range of the metabolized carbohydrates by each yeast of the same chromogenic group, the similarity index (S.I.) was calculated by using the following formula: $S.I. = a/(a + b + c) \times 100$; where a, corresponds to the common characters possessed by both yeasts, b, corresponds to the number of owned characters only from the first yeast, and c, corresponds to the characters possessed only by the second yeast. The isolated type that possessed a 100% similarity and those that were distinguished from the majority of the isolates for the low values of S.I., were chosen for subsequent identification using molecular methods as reported below.

2.4. Identification of the yeast species

The yeasts colony selected above belonging to different chromogenic groups, were subjected to a genetic analysis and identified to species level by sequencing the approximately 600 base-pair D1/D2 region of the large (26S) ribosomal submit using primers NL1 and NL4, as described by Kurtzman and Robnett (1997). This method was developed for the identification of yeast species, and is effective due to identification of this sequence from virtually all

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