International Journal of Food Microbiology 148 (2011) 15–22

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Yeast dynamics during the fermentation of brined green olives treated in the field with kaolin and Bordeaux mixture to control the olive fruit fly

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ARTICLE INFO

Article history:
Received 25 November 2010
Received in revised form 14 April 2011
Accepted 17 April 2011
Available online 22 April 2011

Keywords:
Table olives
Copper
Kaolin
Yeast population
Molecular identification
DGGE

ABSTRACT

The yeast microbiota associated with naturally fermented and inoculated green table olives, differently treated in the field with non-conventional repellent and antiovipositional products in the control of *Bactrocera oleae*, was analysed using a combination of culture-dependent and -independent molecular fingerprinting. The routine yeast isolation gave rise to 118 strains, whose identification was performed by PCR-RFLP of the internal transcribed spacer (ITS) regions. Total DNA was extracted directly from the brine throughout fermentation by means of an experimental protocol that included the removal of Taq polymerase inhibitors. Denaturing Gradient Gel Electrophoresis (DGGE) of 26S rRNA gene PCR amplicons highlighted the yeast community. Comparison of both culture-dependent and independent methods indicated that the yeast species *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Candida diddensiae* and *Issatchenkia orientalis* were dominant during fermentation despite the addition of the *Lactobacillus plantarum* starter used in brining. The resultant isolated species were unaffected by treatments in field, except for *C. diddensiae* whose growth was delayed by kaolin.

1. Introduction

Olives are the major fermented vegetables in western countries (Garrido Fernandez et al., 1997). One of the most detrimental enemies of their quality and processability is the olive fruit fly, *Bactrocera oleae*. It is considered the most devastating insect pest throughout all olive growing regions because its infestation of olive drupes causes a decrease in sensory characteristics and significant losses in phenolic compounds. Alternatives to synthetic chemical pesticides to treat this insect are needed to reduce the impact in the environment and to ensure a safer food supply for consumers. The use of repellent and antiovipositional products, such as kaolin and copper salts, is of great interest in organic farming because of the lack of effective products that are able to kill the olive fly in the pre-imaginal stages (Caleca and Rizzo, 2007). Kaolin forms a protective barrier that controls or suppresses the pest by repelling or irritating the ovipositing females, and copper salts make fruits less attractive to ovipositing females because of the lack of some attractive microbial compounds on the surface of fruits (Belcari et al., 2003; Saour and Makee, 2004).

Homemade production of naturally fermented table olives is very common in Mediterranean countries; the production methods vary according to local tradition, and most often, the process is performed according to an anaerobic method in which the drupes, after harvesting, sorting and washing, are immersed in 8–10% (w/v) NaCl brine, where they undergo spontaneous fermentation by a mixed microbiota of Gram-negative bacteria, lactic acid bacteria and yeasts (Balatsouras, 1990). Lactic acid bacteria and yeasts that coexist throughout fermentation dominate the process; after an initial stage of vigorous fermentation, diverse microbial groups compete for nutrients (Campaniello et al., 2005; Lanciotti et al., 1999; Tassou et al., 2002). Lactic acid bacteria, mainly belonging to *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus brevis* species (Randazzo et al., 2004), influence fermentation in a variety of ways, the most important being the production of lactic acid from fermentable substrates resulting in pH decrease (<4.0), thus ensuring the microbiological stability of the product during storage.

Yeasts play a critical role in all olive fermentations, especially in directly brined green and natural black olives, because the lactic acid bacteria are partially inhibited by the presence of toxic phenolic compounds. The presence of yeast during the fermentation of green olives was reported in the earliest studies of this product (Balatsouras, 1967; González Cancho, 1965). Marquina et al. (1992) and Pelagatti (1978) isolated several species of the genera *Candida*, *Debaryomyces*, *Kluyveromyces*, *Pichia*, *Rhodotorula* and *Saccharomyces* from directly brined green and turning-colour olives. Borcakli et al. (1993) isolated a species of *Debaryomyces* from Turkish cultivars. Kotzekidou (1997) identified *Torulaspora delbrueckii*, *Debaryomyces hansenii* and *Cryptococcus laurentii* as the predominant species in Greek-style black olives, while Marquina et al. (1997), in studies performed with olive brines from seven locations in Morocco, isolated *Candida boidinii*, *Pichia membranifaciens* and *T. delbrueckii*. Hernández et al. (2007) found *Wickerhamomyces anomalus* (formerly *Pichia anomala*), *Kluyveromyces marxianus*, *D. hansenii* and *Saccharomyces cerevisiae* to be the

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doi:10.1016/j.ijfoodmicro.2011.04.019

main species present during the processing of directly brined green table olives from Portugal.

Until recently, the characterisation of yeast associated with table olives has mainly been through biochemical and morphological methods, using the taxonomic keys of Barnett et al. (1990), Kurtzman and Fell (1998) and Looder (1970). More recently, molecular methods, such as PCR-RFLP of 5.8S rRNA-ITSs (Internal Transcribed Spacers), have been employed. Using recent molecular methods, Arroyo López et al. (2006) identified the species *S. cerevisiae*, *Issatchenkia occidentalis* and *Geotrichum candidum* from green seasoned table olives, and *C. boidinii* and *Hanseniaspora guilliermondii* from processed black olives; Coton et al. (2006) identified *W. anomalus*, *C. boidinii* and *Debaryomyces etchellsii* as the predominant species in black olive natural fermentations from France using the same methodology. Finally, Hurtado et al. (2008) found the species *C. boidinii*, *Candida diddensiae*, *Candida membranifaciens*, *Kluyveromyces lactis*, *P. membranifaciens*, *Pichia kluyveri* and *Rhodotorula glutinis* during the processing of Arbequina table olives in Spain, and Nisiotou et al. (2010b) described the novel species *Candida olivae* from "Greek-style" black olive fermentation.

The culture-independent approach is a recent trend in the study of the microbial ecology of foods (Ercolini, 2004). A very recent study of Sicilian olives for microbial detection using different approaches, including a culture-independent methodology, revealed the presence of three yeast species during the entire fermentation period: *Candida parapsilosis*, *Pichia guilliermondii* and *P. kluyveri* (Aponte et al., 2010). However, the polyphasic approach studies that include dependent and independent culture methods, such as DGGE, still need to be performed to broaden our current knowledge of such fermentation.

During the fermentation of table olives, the relevant population of each group depends on several technological factors, such as salt concentration, initial pH adjustment, oxygen availability, the diffusion of nutrients from the drupes and fermentation temperature (Durán Quintana et al., 1986, 1997, 2005; Nychas et al., 2002; Özay and Borcakli, 1996; Tassou et al., 2002). It can also be influenced by treatments used on olives in the fields, such as kaolin and copper salts, to control *B. oleae*; however, no information on their effect on yeast flora of olive oil and table olives is available yet. The effect of copper-containing pesticides on the yeast population has only been investigated in grapes, because copper residues present in musts may cause lagging fermentation and detrimentally affect wine quality (Brandolini et al., 1995; Tromp and De Klerk, 1988).

The aim of this study was to investigate the impact of anti-ovipositional treatments of *B. oleae* on the yeast population of naturally fermented Sicilian green olives. Samples of olives from plants treated with copper or kaolin were analysed for their yeast population from the first day of direct brining up to 180 days of fermentation, using a combination of culture-dependent methods (PCR-RFLP of the ITS regions and D1–D2 sequencing) and the culture-independent method of denaturing gradient gel electrophoresis (DGGE).

2. Materials and methods

2.1. Olive processing

Sicilian green olives, cultivar Nocellara dell'Etna, were treated in field for the control of the olive fruit fly *B. oleae* (Rossi), the key pest of olive groves. The olives were differently treated on trees with a kaolin (Surround® WP-Englelhard Corporation, USA; 3 kg 100 L^{-1} of water) and Bordeaux mixture at 13% (w/w) of copper (Bordolese 13 BP, Manica, Rovereto, Italy; 1.5 kg 100 L^{-1} of water). Both treatments were applied as a single dose at 20 days before the harvest. A crop of untreated olives was used as a control.

Olives were harvested at a maturity stage suitable for processing (mid-October) and selection. Screw-capped PVC vessels (10 kg fruits plus 10 L brine capacity) were filled with olives, and the steps of naturally fermented green olive preparation were performed as follows: (a) washing with water at room temperature; (b) brining, with 8% NaCl; and (c) storing at an environmental temperature (20 °C). For each experimental trial, a crop of olives was inoculated with 1×10^6 cells/mL of the *L. plantarum* $UT_{2.1}$ strain. The experimental design is reported in Table 1.

The pH of the brine solution throughout the fermentation process was determined electrometrically with a pH meter (pH 510, XS Instruments, Giorgio Bormac s.r.l., Carpi (MO), Italy).

2.2. Yeast reference strains

The following strains were used as reference, together with sequenced yeast isolates: *Rhodotorula mucilaginosa* CBS 316, *R. glutinis* CBS 322, *Pichia triangularis* 4094, *Issatchenkia orientalis* DSM 3433, *C. diddensiae* CBS 2214, *S. cerevisiae* DSM 70449, *W. anomalus* CBS 5759, *P. membranifaciens* DSM 70169, and *H. guilliermondii* CBS 465.

2.3. Yeast isolation

Brine samples were collected immediately after brining and at 7, 15, 30, 60, 90, 120, 150 and 180 days of fermentation from each olive lot. Brine samples (1 mL) and appropriate decimal dilutions, in sterile physiologic solution (9 g/L NaCl), were plated on Sabouraud Dextrose Agar supplemented with chloramphenicol (Oxoid, Basingstoke, UK). The plates were incubated at 25 °C for 48–72 h. Approximately 20% of the yeast colonies were randomly selected from plates that showed counts between 30 and 300 colonies, and then purified three times on the same medium. A representative number of 118 yeast isolates were microscopically observed and submitted to molecular identification.

2.4. Yeast identification

2.4.1. DNA extraction from pure cultures

DNA was isolated using the Hoffman and Winston protocol (1987). Pure cultures were grown in YPD medium (g/L of distilled water: Yeast extract 10, Peptone 10, Dextrose 20; Oxoid, Basingtoke, UK) at 25 °C for 18 h at 250 rpm on an orbital shaker. Two millilitres of cell culture was then centrifuged at 9000 *g* for 2 min at room temperature. The cells were resuspended in 400 μL of equilibrated phenol (pH 6), and 400 μL of lysis buffer (10 mM Tris–HCl, pH 8, 10 mM EDTA, 100 mM NaCl, 2% Triton X-100, 1% SDS), and 0.6 g of sterile glass beads (∅ 0.50 mm) (Biospec Products, Bartlesville, OK, USA) was added. The tubes were vortexed for 4 min, centrifuged at 9000 *g* for 1 s and, after adding 200 μL of TE (10 mM Tris–HCl, 1 mM EDTA), the tubes were centrifuged for an additional 5 min at 14,000 *g*. The supernatant was transferred to new tubes; 500 μL of chloroform/isoamyl alcohol (24:1) was added, and the tubes were centrifuged at 14,000 *g* for 2 min. The supernatant was transferred to new tubes with an equal volume of cold isopropanol and stored at −20 °C for at least 30 min. The eppendorf tubes were subsequently centrifuged at 14,000 *g* for 10 min at 4 °C. The pellet was

Table 1
The experimental scheme of the olive fermentations.

Vessel	Treatment in the field	Starter	%NaCl
N1	Untreated	–	6
N2	Untreated	*L. plantarum*	6
N3	Untreated	–	8
N4	Untreated	*L. plantarum*	8
CU1	Copper	–	8
CU2	Copper	*L. plantarum*	8
CU3	Copper	*L. plantarum*	8
CA1	Kaolin	–	8
CA2	Kaolin	*L. plantarum*	8
CA3	Kaolin	*L. plantarum*	8