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Microbiota and metabolome during controlled and spontaneous fermentation of Nocellara Etnea table olives

Cinzia Lucia Randazzo [a, *], Aldo Todaro [b], Alessandra Pino [a], Iole Pitino [a], Onofrio Corona [b], Cinzia Caggia [a]

[a] Department of Agricultural, Food and Environment, University of Catania, Italy
[b] Department of Agricultural and Forest Science, University of Palermo, Italy

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ABSTRACT

This study is aimed to investigate bacterial community and its dynamics during the fermentation of Nocellara Etnea table olives and to study its effect on metabolome formation. Six different combination of bacterial cultures (BC1-BC6) were used as starters for table olive fermentation and one additional process, conducted without addition of any starters, was used as control (C). The processes were conducted in triplicate and, overall, 21 vessels were performed at industrial scale. The fermentation was monitored for 120 days through culture-dependent and −independent approaches. Microbial counts of the main microbial groups revealed slight differences among brine samples, with the exception of LAB counts and *Enterobacteriaceae*, which were higher and lower, respectively, in most of the inoculated samples than the control ones. In addition, results demonstrated that the use of bacterial cultures (except the BC1), singly or in different combinations, clearly influenced the fermentation process reducing the final pH value below 4.50. When microbiota was investigated through sequencing analysis, data revealed the presence of halophilic bacteria and, among lactobacilli, the dominance of *Lactobacillus plantarum* group at the initial stage of fermentation, in all brine samples, except in the BC5 in which dominated *Lactobacillus casei* group. At 60 and 120 days of fermentation, an overturned bacterial ecology and an increase of biodiversity was observed in all samples, with the occurrence of *Lactobacillus paracollinoides*, *Lactobacillus acidipiscis* and *Pediococcus parvulus*. Correlation between bacterial OTU and volatile organic compounds (VOCs) revealed that, aldehydes and alcohol compounds exhibited a positive correlation with Proteobacteria, while several esters with LAB and *Hafnia*. In particular, esters, associated with fruity and floral notes, were positively correlated to *L. paracollinoides, L. acidipiscis*, and *P. parvulus* species. Although the VOCs amounts were sample-specific, overall aldehydes were mostly produced at the beginning of the fermentation, while acids, alcohols and esters at the end of the process.

1. Introduction

Table olives are one of the most produced fermented vegetables in the Mediterranean countries. They are often found available on the market “in bulk”, stored at room temperature, without any thermal treatments (Panagou et al., 2013), since pasteurization is often associated with olive quality deterioration (softening and loss green colour) (Dimou et al., 2013; Abriouel et al., 2014). Thus, the fermentation is the only procedure applied for table olives preservation. It is usually driven by the indigenous microbiota that is strongly influenced by the olive cultivar and technological parameters, such as temperature and salt concentration (Panagou et al., 2003; Heperkan, 2013; Lucena-Padrós et al., 2015). Overall, lactic acid bacteria (LAB) and yeasts represent a house microbiota of table olives fermentation. Nevertheless, members of *Enterobacteriaceae*, *Staphylococcus*, *Pseudomonas* and molds are detected at the beginning of the process, generating off-odours and off-flavours (Romeo, 2102). In addition, table olives are a suitable substrate for growth/survival of pathogens, such as *Clostridium botulinum* and *Listeria monocytogenes*. Several reports on food-borne botulism linked to consumption of conserved olives were already published (Cawthorne et al., 2005; Jalava et al., 2011). Some studies have indicated the survival, even at low concentration, of *Escherichia coli* O157:H7, *Salmonella* Enteritidis, *L. monocytogenes* and *Bacillus*

* Corresponding author.
E-mail address: cranda@unict.it (C.L. Randazzo).

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cereus in the final products (Spyropoulou et al., 2001; Caggia et al., 2004; Argyri et al., 2013). In order to limit the growth/survival of these microorganisms, the use of starter cultures for table olives fermentation is strongly recommended (Aponte et al., 2010; Corsetti et al., 2012). The appropriate use of selected starters can help to achieve a controlled process, reducing debittering time and improving the sensorial and hygienic qualities of the final product (Servili et al., 2006; Panagou et al., 2008; De Angelis et al., 2015; Tataridou and Kotzekidou, 2015). *Lactobacillus plantarum* and *Lactobacillus pentosus* are the most relevant LAB species used as starter cultures, in single or mixed combinations, for natural and treated olives, as reviewed by Heperkan (2013). Moreover, some interaction between LAB and yeasts have already been described, since some yeast species should contribute in the aroma compounds production and/or stimulating the growth of LAB strains (Viljoen, 2006; Hurtado et al., 2011; Arroyo-López et al., 2012; Domínguez-Manzano et al., 2012; Benincasa et al., 2015). Up to now, the application of starter cultures in table olive fermentation at industrial level is still limited. In the present work, we report a comprehensive study on the application, at industrial-scale, of selected starter cultures on the manufacture of Sicilian green table olives belonging to Nocellara Etnea cultivar, with the aim to evaluate their effects on fermentation process, on microbiological quality, and on the volatile organic compounds formation.

2. Material and methods

2.1. Origin of the samples and olive processing

In the present study, olives of Nocellara Etnea cultivar, kindly supplied from a local company, situated in Adrano (Sicily), were processed at industrial scale, following the Sicilian style method, without lye treatment. After harvesting (September–October 2014), olives were calibrated and subjected to grading to remove damaged fruits. Olives were washed with tap water and transferred into 250 l total capacity screw-capped PVC vessels. Each vessel contained proximally 120 kg of olives and 130 l of fresh brine solution. The fermentation trials were carried out at room temperature (ca. 20 ± 2 °C), and monitored for an overall period of 120 days. The brine salt concentration was maintained at initial level of 8% by adding marine salt. Fresh brine was periodically supplied to maintain olives totally dipped in order to inhibit growth of moulds on the brine surfaces.

2.2. Bacterial strains and preparation of inocula

For the preparation of the six different mixtures, three selected strains of LAB, two belonging to the Di3A microbial collection, and one kindly provided from Veneta Agricoltura Company (Istituto per la Qualità e le Tecnologie Agro-Alimentari Thiene, Italy), were used. In detail, the *L. plantarum* UT2.1 and *Lactobacillus paracasei* N24 strains, previously isolated by Randazzo et al. (2007, 2010), were selected for growing at different NaCl concentrations (from 6.0 to 9.0% w v^{-1}), at low pH values (from 3.0 to 5.0), and at low temperatures (10–15 °C), for β-glucosidase activity, for antimicrobial activities and for inability to produce histamine and tyrosine (unpublished data). All cultures were lyophilized from Veneta Agricoltura Company and directly inoculated to reach a final cell density of 7 log colony forming units per ml (CFU ml^{-1}). Overall, six experimental fermentation brines were inoculated with different mixtures of LAB strains described in Table 1. The bacterial culture constituted by the single *L. paracasei* N24 strain was previously tested and was not considered in the present study because negatively influenced the fermentation process (unpublished data). Un-inoculated samples were used as control. Each fermentation process was carried out in triplicate and a total of 21 vessels (18 inoculated and 3 un-inoculated) were obtained.

Table 1
Samples and bacterial cultures used in the present study.

Brine sample	Bacterial cultures
BC1	*L. plantarum* UT2.1 + *L. paracasei* N24 + *L. pentosus* TH969
BC2	*L. plantarum* UT2.1
BC3	*L. paracasei* N24 + *L. pentosus* TH969
BC4	*L. plantarum* UT2.1 + *L. pentosus* TH969
BC5	*L. plantarum* UT2.1 + *L. paracasei* N24
BC6	*L. pentosus* TH969
C	Un-inoculated

2.3. Physico-chemical and total polyphenol determination

Fifty ml of each brine sample were taken after 1, 7, 15, 30, 60, 90 and 120 days of fermentation. During the whole fermentation, brine samples were analyzed for salt concentration by titrating brine samples (5 ml) using a standardized solution of silver nitrate (0.1 N) and potassium chromate (5% w v^{-1}) as indicator (Garrido-Fernández et al., 1997). The pH values of brines were monitored by pHmeter (H19017, Microprocessor, Hanna Instruments). Total polyphenol content was colorimetrically determined in brine samples at 60 and 120 days, using Folin-Ciocalteu reagents, according to the method of Singleton (1974). Polyphenols were measured in triplicate and expressed as mg l^{-1} of gallic acid.

2.4. Microbiological analyses

Microbiological analyses of brine samples were performed after 1, 7, 15, 30, 60, 90 and 120 days of fermentation. At each sampling time, brines were serially diluted, using sterile quarter-strength Ringer's solution, plated in triplicate on the following agar media, all provided from Oxoid (Italy), and incubated at following conditions. Plate Count Agar, incubated at 32 ± 2 °C for 48 h for mesophilic aerobic bacteria count; de Man-Rogosa-Sharp, supplemented with cycloheximide (5 ml l^{-1}), and incubated under anaerobic conditions, at 32 °C for 24–48 h, for LAB count. Sabouraud Dextrose Agar, supplemented with chloramphenicol (0.05 g l^{-1}), incubated at 25 °C for 4 days, for yeast count; Mannitol Salt Agar (MSA, Oxoid), incubated at 32 °C for 48 h, for staphylococci count, and Violet Red Bile Glucose Agar (VRBGA), aerobically incubated at 37 °C for 24 h, for *Enterobacteriaceae* count. Plate count data were reported as $\log_{10}$ values.

2.5. DNA extraction from brine samples

Inoculated brine samples and control samples after 1, 60 and 120 days of fermentation were directly subjected to DNA extraction. In detail, for DNA isolation the *Dneasy Mericon Food* Kit (Quiagen, Milan, Italy) was used, following the instruction procedures with slight modifications. Aliquots (10 ml) of brine samples were centrifuged and the pellet (60 mg) was washed twice with 1× TE buffer (Tris-EDTA) re-suspended in 200 μl of sterile water and 1 ml of *Food Lysis Buffer* plus 10 μl of proteinase K (10 mg ml^{-1}, Sigma) was added. After incubation, with constant shaking, at 60 °C for 40 min, the supernatant was used for DNA extraction using the kit mentioned above.

2.6. Isolation and identification of dominant species throughout recA gene multiplex PCR

A total of 60 colonies were selected from MRS plate of Nocellara