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Co-inoculation approach combining lactic acid bacteria and yeasts to enhance the production of Nocellara del Belice green split table olives

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Abstract:	Table olives are a popular fermented food in the Mediterranean region. In southern Italy, split green table olives are traditionally fermented spontaneously. However, this method poses a risk of product spoilage due to undesirable microorganisms. To address this challenge, driven fermentation using selected starter strains offers a solution, ensuring a safer and more predictable production process. Three distinct experimental productions of Nocellara del Belice split table olives were conducted. In the control trial, a commercial strain of Lactiplantibacillus pentosus OM13 (referred to as OS3) was inoculated individually. In the OS1 and OS2 trials, L. pentosus was co-inoculated with Candida boidinii LC1 and Candida norvegica OC10, previously selected for their bioprotective properties. During the 90-day fermentation process, critical parameters such as pH, salinity and microbial populations were monitored. The olives underwent a comprehensive analysis of colour, pulp texture, volatile organic compounds and sensory traits. During this fermentation, the dominant microorganisms were those deliberately inoculated, mainly lactobacilli and yeasts (> 6 Log CFU/mL). Notably, co-inoculated treatments showed a significant reduction in undesirable microbial populations. Brine acidification occurred rapidly, with pH values reaching 4.5 within 21 days. Overall, this strategic approach that involved fermentation and co-inoculation, which ensured a microbiologically safe product while preserving colour integrity and achieving higher pulp hardness compared to the control trial. The flavour profiles obtained also varied based on the specific inoculum combination, as revealed by sensory analysis, which highlighted significant differences in taste, texture, salinity and overall flavour, without any detectable odours or off-flavours.
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DIPARTIMENTO SCIENZE AGRARIE, ALIMENTARI e FORESTALI

SAAF DIPARTIMENTO SCIENZE AGRARIE ALIMENTARI FORESTALI

Palermo, 08/04/2024

Dear Editor,
I am pleased to submit our paper titled: “Co-inoculation approach combining lactic acid bacteria and yeasts to enhance the production of Nocellara del Belice green split table olives” to your attention.

The aim of this study was to investigate the effect of co-inoculating *Lactiplantibacillus pentosus* with two previously selected yeast strains, *Candida boidinii* and *Candida norvegica*, on the quality of Nocellara del Belice green split table olives processed in the Greek style. The study analysed microbiological, physico-chemical, and sensory parameters, as well as the composition of volatile organic compounds. Co-inoculation of the two yeast strains with *L. pentosus* diversified the aroma of the product and improved its sensory attributes. The most significant impact in co-inoculated trials was the maintenance of drupe colour decay and an increase in pulp hardness.

To our knowledge, no scientific researches have been carried out on these topics.
From recent issues, we believe that our research article may be of interest for Food Bioscience.

The manuscript has been prepared following FBIO authors’ guidelines.

I hope the paper could be revised by FBIO reviewers.

With my best personal regards,
Antonio Alfonzo

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- The co-inoculation of yeast and LAB limited the browning of table olives
- The pulp hardness was higher in table olives that were inoculated with yeasts
- The yeast strains inoculum reduced the population of undesirable microorganisms
- The co-inoculation technique varied the VOC profiles of the trials
- Co-inoculated trials showed improved sensory attributes

Co-inoculation approach combining lactic acid bacteria and yeasts to enhance the production of Nocellara del Belice green split table olives

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ABSTRACT

Table olives are a popular fermented food in the Mediterranean region. In southern Italy, split green table olives are traditionally fermented spontaneously. However, this method poses a risk of product spoilage due to undesirable microorganisms. To address this challenge, driven fermentation using selected starter strains offers a solution, ensuring a safer and more predictable production process. Three distinct experimental productions of Nocellara del Belice split table olives were conducted. In the control trial, a commercial strain of *Lactiplantibacillus pentosus* OM13 (referred to as OS3) was inoculated individually. In the OS1 and OS2 trials, *L. pentosus* was co-inoculated with *Candida boidinii* LC1 and *Candida norvegica* OC10, previously selected for their bioprotective properties. During the 90-day fermentation process, critical parameters such as pH, salinity and microbial populations were monitored. The olives underwent a comprehensive analysis of colour, pulp texture, volatile organic compounds and sensory traits. During this fermentation, the dominant microorganisms were those deliberately inoculated, mainly lactobacilli and yeasts (> 6 Log CFU/mL). Notably, co-inoculated treatments showed a significant reduction in undesirable microbial populations. Brine acidification occurred rapidly, with pH values reaching 4.5 within 21 days. Overall, this strategic approach that involved fermentation and co-inoculation, which ensured a microbiologically safe product while preserving colour integrity and achieving higher pulp hardness compared to the control trial. The flavour profiles obtained also varied based on the specific inoculum combination, as revealed by sensory analysis, which highlighted significant differences in taste, texture, salinity and overall flavour, without any detectable odours or off-flavours.

Keywords: Bioprotection; *Candida boidinii*; *Candida norvegica*; Fermentation; Greek style; Table olives

1. Introduction

Table olives constitute a typical fermented food in the Mediterranean basin (Perpetuini et al., 2020). Over the last five years (2016-2021), the global average production reached approximately 3 million tonnes, with EU countries contributing 847,000 t (IOC, 2023). Among these countries, Spain stands out as the primary EU producer, closely followed by Greece, Italy and Portugal. Green table olives undergo various processing methods, all aimed at reducing bitterness from the drupes (Sánchez et al., 2021). The compound responsible for this bitterness is oleuropein, a polyphenol. Its concentration depends on the variety and maturity of the olives (Conte et al., 2020). Preedy and Watson (2020) have described two methods for oleuropein hydrolysis: chemical or biochemical.

The Castelvetrano method involves chemical debittering, while the Greek or natural style relies on biochemical hydrolysis of oleuropein (Ambra et al., 2017; Boskou et al., 2015). In the production of Seville-style table olives, oleuropein is first chemically hydrolysed using soda treatment, followed by enzymatic hydrolysis through beta-glucosidase activity of microorganisms (Boskou et al., 2015). In Italy, the Sevillian and natural processing styles diverge from the original protocols, influenced by regional customs and traditions.

In Sicily, the southernmost region of Italy, a portion of the locally available table olives undergoes transformation using the Greek or natural method. Before fermentation, these olives undergo a preliminary treatment. The drupes are longitudinally cut, creating a slit in the fruit's skin down to the level of the stone. In some variations, the olives are crushed instead of incised, a method widely practiced in various olive-producing regions (Kailis and Harris, 2007). This process mainly utilizes clingstone olive varieties such as Bella di Cerignola, Carolea, and Frantoio. However, in Sicily, split olives are significantly produced using the freestone variety Nocellara del Belice. The fermentation process, which is predominantly uncontrolled, occurs through the activity of indigenous lactic acid bacteria (LAB) and yeasts present on the drupes. These microorganisms play a crucial role in acidifying the brine, essential for limiting the presence of altering microorganisms and potential pathogens that could compromise the safety and quality of the final product (Hurtado et al., 2008).

However, spontaneous fermentation carries risks, resulting in annual product losses due to unpleasant odours and flavours caused by the metabolism of undesirable microorganisms (Valero et al., 2016). Producers may also observe softening of the drupes and depigmentation of the final product. Alves et al. (2012) emphasize the importance of using specific starter cultures to enhance the quality of cracked green table olives, particularly in terms of standardizing the product's quality. Additionally, a rapid acidification process should create an unfavourable environment for various food pathogens that might potentially survive in cracked table olives (Medina et al., 2016).

Recently, several authors have underscored the advantages of employing mixed-culture starter strains of yeasts and LAB to enhance the fermentation process, thereby improving the chemical, physical, and sensory characteristics of table olives (Benítez-Cabello et al., 2020a; Chytiri et al., 2020). In their study, Benítez-Cabello et al. (2019) highlighted the significance of using yeasts in combination with LAB during the fermentation of table olives. Regarding aromaticity, yeasts contribute to the production of volatile organic compounds. However, the inoculation of LAB starters, selected primarily for their acidifying capacity, results in an aromatic profile very similar to, if not less favorable than, that of olives produced through spontaneous fermentation. To enhance the quality of this product, green split table olives of the Nocellara del Belice variety were produced using a commercial starter strain, *Lactiplantibacillus pentosus* OM13, widely employed on an industrial scale (Alfonzo et al., 2023a; Martorana et al., 2017). In additional experimental productions, *L. pentosus* OM13 was co-inoculated with two yeast strains, *Candida boidinii* LC1 and *Candida norvegica* OC10. These strains were previously characterized for their bioprotective activity (Alfonzo et al., 2024). The fermentation process was monitored from both microbiological and physicochemical aspects. The suitability of the LAB starter strain and the yeasts used in co-inoculation was evaluated by assessing the aromatic and sensory qualities of the final product.

2. Materials and methods

2.1. Experiment model, table olive production process and sampling

The Nocellara del Belice drupes were harvested from olive groves in Campobello di Mazara (Trapani, Italy) and generously donated by Alongi s.n.c., owned by Alongi Girolamo & Roberto (Villabate, Italy). Prior to fermentation, the drupes with deformities, browning, or necrosis were discarded, and only drupes belonging to the extra-large category were selected for the experiments. The olives were prepared using a mechanical cutting machine (Ip Bantli Zeytin Çizme Makinesi, Günal Makina, Havran, Balıkesir, Turkey). This machine made longitudinal cuts up to the stone of the olives. Subsequently, the drupes were promptly transported to the Agricultural Microbiology Laboratories of the Department of Agricultural, Food and Forest Sciences at the University of Palermo. A total of 75 kg of Nocellara del Belice table olives were divided into 9 glass jars, each with a capacity of 10 L. The jars were filled with 8.3 kg of drupes and 1.7 L of brine containing 8% (w/v) NaCl. Three treatments were performed (Fig. 1): (i) co-inoculation (OS1), Lal'Olive Crispy *L. pentosus* OM13 (Lallemand Inc., Castel D'Azzano, Italy) was combined with *C. boidinii* LC1 in liquid concentrated form [approximately 7.00×10^{10} colony forming units (CFU)/mL; Bionova srl, Villanova sull'Arda, Italy]; (ii) co-inoculation (OS2), Lal'Olive Crispy *L. pentosus* OM13 (Lallemand Inc., Castel D'Azzano, Italy) was combined with *C. norvegica* OC10 in liquid concentrated form (approximately 7.00×10^{10} CFU/mL; Bionova srl]; (iii) inoculation (OS3), Lal'Olive Crispy *L. pentosus* OM13 (Lallemand Inc., Castel D'Azzano, Italy) in freeze-dried form (containing approximately 10^9 CFU/g) with food-grade maltodextrin as a carrier. The dosages for the starter strain was 0.0083 g of freeze-dried cells of Lal'Olive Crispy *L. pentosus* OM13 per Kg olives + brine. For the two yeast strains, the dosage used was 2 g per 10 kg of olives. After inoculation, the drums were sealed, coded, and placed in a room at 22 ± 1 °C. The sampling occurred at various time points (0, 7, 21, 60, 75, and 90 days) with olives collected in brine (~ 200 mL) for analysis.

2.2. Physicochemical parameters

111 The pH of the brine was determined following the procedure described by Alfonzo et al.
112 (2023b). Brine salinity, expressed as % NaCl, was measured using a digital refractometer DBS1
113 (Giorgio Bormac srl, Carpi, Italy) as described by Sidari et al. (2019). These parameters were taken
114 at specified sampling intervals, and each value represents the average of three replicates.
115 At the 90-day mark, a Chroma Meter CR-400C (Minolta, Osaka, Japan) was used for
116 colorimetric measurements of the drupes, according to the methodology of Martín-Vertedor et al.
117 (2022). The color space was characterized using three parameters: brightness (L), which ranges from
118 0 (black) to 100 (white); red/green variation (a*), where negative values indicate green and positive
119 values indicate red; and yellow/blue variation (b*), where positive values indicate yellow and
120 negative values indicate blue. Thirty randomly selected olives were used to measure each parameter.
121 After 90 days, 30 drupes were randomly sampled for each treatment. The pulp hardness,
122 expressed in kg/cm^2, was assessed using a FT327 portable penetrometer (Facchini srl, Alfonsine,
123 Italy) according to Kaya et al. (2017). The average of 30 measurements taken from randomly chosen
124 olives was used to determine pulp hardness.
125
126 *2.3. Microbiological analysis*
127 The brine samples underwent serial dilution in a 1:10 ratio using Ringer's solution (Liofilchem
128 srl, Roseto degli Abruzzi, Italy). To enumerate the monitored microbial groups, the following media
129 were employed: Plate Count Agar (PCA) for total mesophilic microorganisms, incubated for 2 d at
130 30 °C (Tofalo et al., 2012); de Man-Rogosa-Sharpe (MRS) supplemented with cycloheximide (10
131 mg/mL) for LAB rods and incubated anaerobically for 2 d at 30 °C (Alfonzo et al., 2023c); dichloran
132 rose bengal chloramphenicol (DRBC) agar for yeasts, incubated aerobically at 25 °C for 5 d
133 (Martorana et al., 2015); violet red bile glucose agar (VRBGA) for Enterobacteriaceae, incubated
134 aerobically for 1 d at 37 °C (Martorana et al., 2015); Violet Red Bile Agar (VRBA) for coliforms,
135 incubated aerobically for 1 d at 37 °C (Tufariello et al., 2019); Hektoen enteric agar (HEA) for
136 *Salmonella* spp. and *Shigella* spp., incubated aerobically for 1 d at 37 °C (Busetta et al., 2023);

mannitol salt agar (MSA) for Staphylococcaceae, incubated aerobically for 2 d at 30 °C (Anagnostopoulos et al., 2019); Pseudomonas agar with CFC supplement (PCFC) for Pseudomonadaceae, incubated aerobically for 2 d at 30 °C (Aponte et al., 2012). These analyses were conducted in triplicate using media and supplements provided by Condalab (Torrejón de Ardoz, Spain).

2.4. Dominance of inoculated strains

For each trial and sampling point, isolates were obtained from the highest dilutions of the respective culture medium (DRBC and MRS) to verify the dominance of *C. boidinii* LC1, *C. norvegica* OC10, and *L. pentosus* OM13. At least five colonies from each yeast group or LAB, exhibiting different macroscopic characteristics (such as colour, edge, elevation, shape, and width) were purified to obtain axenic cultures. Cell morphology was assessed by observing samples under the Axiophot light microscope (Carl Zeiss, Oberkochen, Germany). Presumptive LAB were identified based on Gram's staining (Gregersen, 1978) and the presence or absence of the enzyme catalase (Reniner, 2010). DNA extraction was carried out on 30% of the yeast and LAB isolates within each phenotype group using InstaGene Matrix kits (Bio-Rad, Hercules, CA, USA; Alfonzo et al., 2013; Alfonzo et al., 2021). To assess the dominance of the inoculated strains, the random amplification of polymorphic DNA-PCR (RAPD-PCR) with primer M13 was applied. The protocol for yeasts followed that described by Andrighetto et al. (2000), while for LAB, it adhered to the procedure reported by Rossetti and Giraffa (2005). The dominance percentage for each trial was calculated by comparing the polymorphic profiles of the inoculated strains with those of the isolates collected during the fermentation process. These RAPD profiles were visualized and compared using the approach detailed by Alfonzo et al. (2023b).

2.5. Volatile organic compounds

Headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS) was used to analyse and identify esters and higher molecular weight alcohols in olive samples. Volatile organic compounds (VOCs) were analysed in olive samples before the fermentation process began, immediately after immersion in brine (OBF), and at the end of the 90-day process in the three treatments (OS1, OS2 and OS3) described above. Standard organic compounds were injected throughout the experimental sequence using the identical extraction procedure to detect and evaluate each compound. The identification of each compound was facilitated by the use of Kovats indices (KI). KI values are based on the retention time normalised to the adjacent eluted n-alkanes. KI values are independent of the method of analysis. They are therefore useful for the identification of unknown compounds. An Agilent 5890 GC system coupled to an HP 5973 quadrupole mass spectrometer was used for the gas chromatographic analyses. An HP5-MS column (5% diphenyl - 95% dimethyl polysiloxane 30 m×0.2 mm, 0.25 µm film, J & W Scientific, Folsom CA, USA) was used. Water and oxygen traps (Supelco, ITA) were installed on the carrier gas lines and ultra-high purity helium was used as the carrier gas at a flow rate of 1 mL/min. The oven temperature was maintained at 40 °C for five minutes, then increased at a rate of 5 °C/min to 220 °C and 10 °C/min to 280 °C, and held constant for 10 minutes. Carrier gas (He) was used at a flow rate of 1 mL/min. Molecular mass spectra were recorded using an ionisation voltage of 70 eV and an ion source temperature of 220 °C (Catania et al., 2016). The samples were analysed by the HS-SPME-GC-MS method using a PDMS-CAR-DVB fibre (Supelco, ITA). The process of extracting and purifying the material involved exposing the fibre to three pieces of thinly sliced olive (3 g) in a 40 ml vial with a silica septum at 70 °C for 20 minutes. Fiber was manually inserted into a GC inlet port equipped with a specific glass liner for SPME injection (0.75 mm i.d.). Fibers were removed from the gas chromatograph inlet port by using splitless injection mode for 3 minutes at 250 °C. The compounds were confirmed by comparison of the mass spectral data with those of authentic reference compounds. When standards were unavailable, the identification of the components was conducted via mass spectrum matching utilizing the NIST11 mass spectral library collection.

188

189 *2.6. Sensory analysis*

190 Each treatment underwent evaluation by a panel of 16 trained tasters (8 men and 8 women
191 aged between 27 and 58) using the methodology established by the International Olive Oil Council
192 (Regulation COI/OT/MO No 1/Rev.2). To remove excess salt, each experimental sample was rinses
193 three times with low-mineral water before the tastings. Subsequently, each sample, labelled with a 5-
194 digit alphanumeric code, was served to the judges in ceramic plates. The sensory profile of the green
195 split table olives was evaluated based on seven attributes: texture, flavour, salinity, bitterness, acidity,
196 off-flavours and general evaluation (Anagnostopoulos et al. 2019; Anagnostopoulos et al., 2020). The
197 tasting session was conducted in well-lit rooms at an ambient temperature of 23 ± 1 °C. To cleanse
198 their palate between sampling the different olive samples, the tasters were offered water and salt-free
199 crackers. Each attribute was evaluated on a scale of 0 to 10, following the methodology of
200 Anagnostopoulos et al. (2019).

201

202 *2.7. Statistical analysis*

203 The growth dynamics of the monitored microbial populations, chemical-physical parameters (pH, salt
204 content of the pulp and brine, pulp colour and hardness), and sensory analysis were analysed using
205 ANOVA and pairwise comparisons with Tukey's test for $P < 0.001$. The treatments were grouped
206 based on their aroma profile using Agglomerative Hierarchical Clustering (AHC). To perform this
207 analysis, we considered the number of VOCs and their respective relative peak area percentage
208 values. We measured the dissimilarity between the treatments using euclidean distances and Ward's
209 method. All analyses were conducted using XLStat software version 2019.2.2 (Addinsoft, New York,
210 USA).

211

212 **3. Results and discussion**

213 *3.1. pH and salinity*

214 The acidification process in the brine followed a consistent pattern as fermentation progressed.
215 During the initial stages of fermentation, there was a significant drop in pH values, which continued
216 until the 7th day. In all treatment groups (Table 1), the pH values fell below 5.0. The rapid pH decrease
217 is commonly observed in split or cracked olives. Similar trends have been documented for both split
218 table olives, such as Cobrançosa (Prata et al., 2019), and cracked table olives, such as Maçanilha
219 Algarvia (Mateus et al., 2016; Saúde et al., 2017). The OS3 trial exhibited the lowest pH values from
220 day 21 until the end of fermentation. By the end of the monitoring period for all experimental
221 treatments, the pH remained below 4.5. This pH level ensures the safety of table olives by preventing
222 the growth of harmful microorganisms during storage (Tofalo et al., 2012). Specifically, the pH
223 values were recorded as 4.00 in OS3, 4.19 in OS2, and 4.31 in OS1. Although these values indicate
224 microbiological safety, the trials involving the yeast-LAB co-inoculum (OS1 and OS2) showed
225 slightly slower acidification compared to the control trial (OS3). These subtle differences may be
226 attributed to the higher levels of inoculated yeasts, which had a minor impact on the acidifying
227 capacity of *L. pentosus* OM13 (Hurtado et al., 2012; Panagou et al., 2008).

228 During the fermentation of table olives, the monitoring of salt content in both the brine and pulp
229 is essential. This is because salt directly influences the activity of microorganisms participating in the
230 fermentation process (Botta and Cocolin, 2012). Initially, the brine contained approximately 8% salt,
231 while the pulp had a salt content ranging from 7.00 to 7.30%. As fermentation progressed, the salt
232 content in the pulp increased, while the brine salt content slightly decreased. Variations in salt content
233 were observed between treatments in both the pulp (at 0 and 60 days) and the brine (at 60 and 90
234 days), which is a normal occurrence (Prata et al., 2019; Simone et al., 2020). However, after 90 days,
235 the salt content reached an equilibrium state, averaging 8%. The pH values and salt content recorded
236 after 90 days should ensure the microbiological safety of the product by limiting the growth of
237 undesirable microorganisms (Kailis and Harris, 2007).

238

3.2. Physical parameters

The colour of table olives is primarily influenced by their degree of ripeness and the processing style (Lanza and Ninfali, 2020). Greek-style table olives exhibit a brownish hue, while Seville-style table olives lean towards a yellowish colour, which tends to be more visually appealing to consumers (Rocha et al., 2020). The difference in colour arises from a series of oxidative processes that enzymatically break down o-diphenolic compounds. In Seville-style table olives, enzymatic reactions are inhibited by the use of sodium hydroxide during partial debittering of the drupes (Ramírez et al., 2015). Table 2 presents the results of olive colour analysis. Notably, there were no significant differences in lightness (L) among the various treatments. This parameter appears to be largely unaffected by processing, as previously observed by Romeo et al. (2012), but it mainly reflects varietal characteristics (Pires-Cabral et al., 2018). Regarding the parameter a*, in contrast to Romeo et al. (2012) findings for whole and cracked olives, the natural-style olives in our study exhibited negative values across all treatments. Specifically, trials OS1 and OS2 showed lower values than OS3, indicating a higher intensity of green color in the experimental trials inoculated with yeasts, consistent with data of Alfonzo et al. (2024). As for the b* parameter, the OS3 control trial had a lower value (33.17), while the highest value was recorded in OS2 (36.94). The OS2 treatment resulted in higher values, suggesting less browning of the drupes. Interestingly, our recorded data exceeded those reported in the literature for Nocellara del Belice (Romeo et al., 2012), Manzanilla, and Hojiblanca (Ramírez et al., 2015) in table olives produced in a natural style. These values were comparable to those reported by Alfonzo et al. (2024) for Nocellara del Belice olives debittered using the soda method (Castelvetrano method).

In terms of pulp hardness, both the OS1 and OS2 trials exhibited higher values compared to the control trial, which was exclusively inoculated with *L. pentosus* OM13. This result underscores the ability of the inoculated yeasts (*C. boidinii* LC1 and *C. norvegica* OC10) to create an environment

263 that discourages the presence of pectinolytic and cellulosolytic microorganisms responsible for
264 softening the pulp (Golomb et al., 2013; Kailis and Kiritsakis, 2017).
265
266 *3.3. Dynamics of microbial populations monitored*

267 Figure 2 illustrates the trend data for the monitored microbiological groups over the 90-day
268 period. The highest levels of total mesophilic microorganisms occurred after 21 days in both OS1
269 (8.1 Log CFU/mL; Fig. 2a) and OS2 (7.8 Log CFU/mL; Fig. 2b). However, from day 60 until the end
270 of the monitoring period, the concentration of total mesophilic microorganisms gradually decreased
271 by 0.1 and 1.1 log cycles. Interestingly, microbial densities recorded at 75 and 90 days in OS1 and
272 OS2 were higher compared to the control trial OS3. This phenomenon might be attributed to the
273 greater yeast presence in the trials involving the inoculation of *C. boidinii* and *C. norvegica*. In
274 comparison to data reported by Pires-Cabral et al. (2018), which predicted spontaneous fermentation
275 of cracked and split olives, the values recorded in our experiment were 2-3 log cycles higher.
276 Specifically, yeast levels were higher in OS1 (7.3 ± 0.6 Log CFU/mL; Fig. 2a) and OS2 (7.2 ± 0.5
277 Log CFU/mL; Fig. 2b) compared to the OS3 control treatment (5.1 ± 0.9; Fig. 2c), indicating a
278 significant difference. The gap between inoculated and non-inoculated yeast trials was 2.7-3.0 log
279 cycles after one week, which decreased to 1 log cycle by day 90 (Fig. 2). Benítez-Cabello et al.
280 (2020a) observed a comparable trend in Spanish green table olives inoculated with *Wickerhanomyces*
281 *anomalus*. The inoculated and non-inoculated trials showed a difference of approximately 2 log
282 cycles. Yeast inoculation did not significantly affect lactobacilli levels during the initial 60 days of
283 fermentation. In fact, the differences observed between treatments were minimal. The highest
284 lactobacilli populations were observed on day 21, ranging from 8.0-8.2 Log CFU/mL (Fig. 2), with a
285 slight decrease in subsequent monitoring points. However, the lactobacilli population remained
286 higher in the OS1 and OS2 treatments, which involved yeast inoculation, even on days 75 and 90.
287 This is likely due to the presence of a high density of yeast population, which can increase the

availability of growth factors and promote the growth of lactobacilli, as reported by Hurtado et al. (2012), Psani and Kotzekidou (2006), and Mougiou et al. (2023).

On day 0, both OS1 and OS3 showed the presence of Enterobacteriaceae and coliforms (2.1 and 1.1 Log CFU/mL, and 2.1 and 2.0 Log CFU/mL, respectively; Fig. 2a and 2c). After 7 days, only OS1 had levels slightly above the detection limit (1.2 Log CFU/mL). In the OS2 trial, Enterobacteriaceae and coliform populations remained undetected at all sampling times (Fig. 2b). The reduction in pathogenic microorganisms is attributed to the rapid acidification of the brine caused by the starter strain *L. pentosus* OM13. This trend was also observed by Anagnostopoulos et al. (2019) in green cracked Cypriot table olives, where both Enterobacteriaceae and coliforms were significantly reduced after two weeks, coinciding with the decline in brine pH values (Benítez-Cabello et al., 2023). Notably, *Salmonella* spp. and *Shigella* spp. were detected only at the onset of fermentation (day 0) in OS1 (2.1 Log CFU/mL) and OS3 (1.7 Log CFU/mL). It is worth mentioning that all colonies counted exhibited a green color and were morphologically identified as *Shigella* spp. (Gaurav et al., 2013).

The microbial densities of Staphylococcaceae decreased in all treatments (Fig. 2a, b, c). In the OS2 trial, which was inoculated with *C. norvegica*, the lowest microbial densities of Staphylococcaceae were observed, ranging from 4.0 Log CFU/mL (at day 0) to 2.2 Log CFU/mL (at day 90). Comparatively, in OS1 and OS3, microbial levels were higher by approximately 1 log cycle. By day 90, the levels of Staphylococcaceae populations in OS1 and OS2 converged to 2.2 Log CFU/mL, while in the control treatment OS3, they remained slightly above 4 log cycles. Throughout the monitoring period and across all treatments, yellow halo colonies attributable to *Staphylococcus aureus* were not observed in the MSA (Kateete et al., 2010). Staphylococci are a group of microorganisms commonly found in table olives, serving as indicators of the product hygienic status. Due to their physiological characteristics, they exhibit resistance to pH, temperature, and salinity, and are usually present during both the early stages of fermentation and in the final product (Benítez-Cabello et al., 2020b). The inoculation of *C. norvegica* seems to have impacted the levels of

staphylococci in OS2, a phenomenon also observed in Nocellara del Belice table olives produced using the Castelvetrano method (Alfonzo et al., 2024).

The levels of pseudomonads observed in the different treatments showed distinct trends (Fig. 2a, b, c). Specifically, in OS1, there was a gradual decrease over time [from 4.4 Log CFU/mL (at day 0) to 2.2 Log CFU/mL (at day 90]. In OS2, pseudomonad levels decreased from 3.1 Log CFU/mL (at day 0) to 2.1 Log CFU/mL (at day 60), with values falling below the detection limit on subsequent days (at 75 and 90 days). Throughout the monitoring period, the trend of pseudomonadaceae in OS3 fluctuated slightly about 3 log cycles. Comparing the three treatments, in OS1, the highest counts were recorded up to day 60, whereas in OS3, the highest counts occurred from day 60 until the end of monitoring. In contrast, in OS2, the pseudomonad populations consistently remained lower than those in OS1 and OS3. Remarkably, only the levels of pseudomonad in OS2 were comparable to those reported by Mateus et al. (2016) for cracked table olives produced using a similar process. The low count values recorded in the other treatments should not cause any deterioration (Alfonzo et al., 2024). It is worth noting that Pseudomonads could potentially pose a problem in olives, as they indirectly contribute to the production of biogenic amines in table olives due to their ability to produce such compounds (Kazou et al., 2020).

3.4. Dominance of inoculated strains of yeasts and lactic acid bacteria

A total of 498 yeast isolates and 721 presumptive LAB isolates were collected. Specifically, 136 yeast isolates were obtained from OS1, 238 from OS2, and 124 from OS3. Among the 721 LAB isolates, 614 were identified as LAB due to their Gram-positive nature and catalase negativity. These LAB isolates were distributed across the treatments as follows: 218 from OS1, 173 from OS2, and 223 from OS3. Using RAPD-PCR analysis, 12 polymorphic profiles for yeasts and 8 for LAB were identified. These profiles exhibited distinct distributions based on the treatments (Fig. 3). In OS1, 85.3% of the isolates displayed a polymorphic profile identical to the inoculated yeast strain *C.*

boidinii LC1 (Fig. 3a). Similarly, the starter strain *L. pentosus* OM13 also exhibited the same percentage of dominance (Fig. 3b). In OS2, the yeast strain *C. norvegica* OC10 had the dominant RAPD profile (86.6%), while *L. pentosus* OM13 showed slightly lower dominance (83.8%) compared to the OS1 trial. Interestingly the OS3 trial did not reveal any polymorphic profile corresponding to the yeast strains *C. boidinii* LC1 and *C. norvegica* OS10. However, the dominance of *L. pentosus* OM13 was higher (89.2%) in OS3 compared to both OS1 and OS2 trials. Similar trials conducted on Nocellara del Belice olives, but obtained through the Seville processing style, showed comparable dominance percentages of *L. pentosus* OM13 (Alfonzo et al., 2023a, b, c). Additionally, Chytiri et al. (2020) studied the production of Greek-style table olives in Kalamàta and Conserveola. They reported similar percentages of LAB starter cultures and inoculated yeasts when co-inoculation was used, although the dominance percentages of inoculated starter cultures were lower compared to those obtained in this trial. Notably, the percentage dominance of the inoculated strains is influenced by fermentation conditions, table olive varieties, and drupe incision.

3.4. VOCs composition

Chromatographic analysis was performed on the headspace of various experimental productions at two different times: before fermentation (OBF) and after 90 days of fermentation (OS1, OS2 and OS3). A total of 63 compounds were detected, which were distributed differently depending on the treatments (Fig. 4). Twenty-three compounds were detected in the unfermented olives (OBF), 27 in trial OS1, 29 in trial OS2, and 34 in trial OS3. Across all fermented trials, Cresol had the highest relative peak area values, followed by Phenol-4-ethyl-. The volatile phenols are primarily generated by lactobacilli (*L. plantarum* and *L. pentosus*) from hydroxycinnamic acids and the amounts produced is strain-dependent, but also depend on table olive variety (Ruiz-Barba et al., 2023a). However, n-decanoic acid was the compound with the highest relative peak area percentages both before and after the fermentation process. After 90 days, the OS2 (16.2) and OS3 (13.4) trials showed an increase in

363 the percentage values of relative peak area compared to OBF (9.1). On the contrary, OS1 trial showed
364 a decrease (3.9). The decrease in n-decanoic acid is typically associated with the fermentation of
365 Greek-style table olives (Ruiz-Barba et al., 2023b). In OBF, the compound with the highest relative
366 peak area values was 3-Hexen-1-ol, which was not detected in any of the fermented treatments. This
367 aligns with the observation of the unfermented Campo Real table olives (Navarro et al., 2004). So
368 far, the detection of 3-Hexen-1-ol in unfermented Nocellara del Belice table olives has never been
369 reported. Among terpenes, copaene exhibited a higher relative peak area percentage in OBF (18.1%);
370 after 90 days, this compound was only detected in OS1 trial (8.8%). This observation is in contrast
371 with the findings of Sánchez et al. (2018), who registered a significant decrease of the amounts of
372 copaene after fermentation. Additionally, octanoic acid is a compound found at high concentrations
373 in Nocellara del Belice olives (Aponte et al., 2010). The relative peak area values were highest in
374 OBF (9.4), while a decrease in this compound was observed in all treatments after fermentation, from
375 4.3 for OS3 to 3.3 for OS1. This decrease is due to the conversion of octanoic acid into its respective
376 esters during fermentation (Cortés-Delgado et al., 2016). Remarkably, only 11 compounds were
377 detected in the olives before fermentation took place (OBF), while 4 compounds were detected in
378 OS1, 6 compounds in OS2, and 9 in OS3 (Fig. 4). The AHC (Fig. 4) allowed to differentiate among
379 the various treatments based on the number and percentage value of relative peak area of each
380 compound. At a 5% dissimilarity, fermented olives (OS1, OS2, and OS3) and unfermented olives
381 (OBF) formed four distinct clusters. The variables that significantly influenced the clustering of the
382 trials were 3-Hexen-1-ol for OBF, phenylethyl alcohol for OS1, .alpha.-Cubebene for OS2, and
383 .alpha.-Copaene for OS3. Phenylethyl alcohol might be produced by *C. boidinii*, as well as other yeast
384 species (Montaño et al., 2021). Alpha.-Cubebene and .alpha.-Copaene are sesquiterpenes present in
385 various table olive cultivars produced with different production styles (Alfonzo et al., 2023b; Bleve
386 et al., 2015; Dabbou et al., 2012).
387
388 *3.5. Sensory evaluation*

The sensory profiles of the split table olives are illustrated in Fig. 5. The attributes of acidity, bitterness, and off-flavours received similar scores across all treatments. However, significant differences were observed for the attributes of flavours, texture, salty, and general evaluation. Specifically, OS2 obtained the highest scores for flavour and texture, while the lowest scores were observed in the OS3 control trial. Salty was highest in OS1 and lowest in OS2. In terms of overall evaluation, trials OS1 and OS2, which were co-inoculated, received the highest scores. Notably, the panelists preferred the trial involving co-inoculation of *C. norvegica* and *L. pentosus* (OS2). It is important to recognize that the co-inoculation technique, which involves LAB and selected yeasts may produce varying results in Greek-style table olives of different varieties, such as Bella di Cerignola, Conserveola, and Kalamata. The outcome of the fermentation process depends on the combination of LAB-yeast strains (De Angelis et al., 2015) and the specific olive variety being fermented (Chytiri et al., 2020). Remarkably, the product inoculated with both yeast and LAB exhibits a superior sensory profile in terms of general evaluation and aromaticity.

4. CONCLUSIONS

Split table olives, although available in smaller quantities compared to whole table olives, pose several challenges related to the production process. Two common issues associated with this type of product are softening of the pulp and browning of the drupes. The softening phenomenon primarily arises from the enzymatic activity of indigenous yeasts during the fermentation process. While using a LAB starter strain can enhance the final product, it does not always provide a satisfactory solution. In this specific case, the problem was addressed by co-inoculating *L. pentosus* OM13 with either *C. boidinii* LC1 or *C. norvegica* OC10. The addition of yeasts has proven to be an effective strategy for preventing browning. Greek-style table olives experience more pronounced oxidative processes than Seville-style olives. Consequently, employing yeasts capable of counteracting this phenomenon could be a viable industrial approach in the future. Co-inoculating the LAB starter strain with yeasts does not disrupt fermentation dynamics, allowing the prompt acidification of the brine. Furthermore, the

co-inoculation technique establishes a controlled environment that restricts the growth of undesirable microorganisms during the initial fermentation days. An analysis of VOCs revealed that the co-inoculation strategy (yeasts/LAB) leads to a diversification of aroma profiles, as confirmed by sensory evaluations. Utilizing yeasts with bioprotective properties holds promise for enhancing the quality of split table olives, resulting in a product that is both microbiologically safe and aligned with current market demands.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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433 **Table 1**

434 Changes in pH and salt content of the brine and pulp of green split Nocellara del Belice table olives.

Days	pH			S.S.	Pulp salt content (%)			S.S.	Brine salt content (%)			S.S.
	OS1	OS2	OS3		OS1	OS2	OS3		OS1	OS2	OS3	
0	6.24 ± 0.04^{a}	6.35 ± 0.06^{a}	6.31 ± 0.05^{a}	n.s.	7.30 ± 0.10^{a}	7.00 ± 0.10^{b}	7.20 ± 0.05^{a}	*	8.30 ± 0.10^{a}	8.40 ± 0.10^{a}	8.40 ± 0.10^{a}	n.s.
7	4.98 ± 0.05^{a}	4.64 ± 0.04^{b}	4.58 ± 0.06^{b}	*	7.90 ± 0.10^{a}	7.80 ± 0.10^{a}	7.80 ± 0.05^{a}	n.s.	8.10 ± 0.10^{a}	8.10 ± 0.10^{a}	8.10 ± 0.10^{a}	n.s.
21	4.84 ± 0.05^{a}	4.58 ± 0.06^{b}	4.43 ± 0.06^{c}	*	8.00 ± 0.05^{a}	8.10 ± 0.10^{a}	8.20 ± 0.05^{a}	n.s.	6.90 ± 0.10^{a}	6.80 ± 0.10^{a}	7.20 ± 0.20^{a}	n.s.
60	4.66 ± 0.05^{a}	4.38 ± 0.03^{b}	4.26 ± 0.05^{c}	**	8.60 ± 0.10^{a}	8.50 ± 0.05^{a}	7.90 ± 0.15^{b}	*	7.70 ± 0.10^{b}	7.40 ± 0.10^{c}	8.00 ± 0.05^{a}	*
75	4.48 ± 0.05^{a}	4.25 ± 0.04^{b}	4.01 ± 0.04^{c}	**	8.20 ± 0.05^{a}	8.10 ± 0.10^{a}	8.00 ± 0.10^{a}	n.s.	8.00 ± 0.10^{a}	8.10 ± 0.10^{a}	8.00 ± 0.10^{a}	n.s.
90	4.31 ± 0.05^{a}	4.19 ± 0.05^{a}	4.00 ± 0.04^{b}	*	8.10 ± 0.10^{a}	8.00 ± 0.20^{a}	8.20 ± 0.10^{a}	n.s.	7.90 ± 0.05^{b}	8.00 ± 0.05^{ab}	8.10 ± 0.05^{a}	*

435 Result indicate mean value (± standard deviation).

436 Abbreviations: OS1, *Lactiplantibacillus pentosus* OM13 + *Candida boidinii* LC1; OS2, *L. pentosus* OM13 + *Candida norvegica* OC10; OS3, *L. pentosus* OM13 (control); n.s.,
437 not significant.

438 Data in the same line followed by the same letter are not significantly different according to Tukey's test. P value: **, $P < 0.01$; *, $P < 0.05$.

Table 2

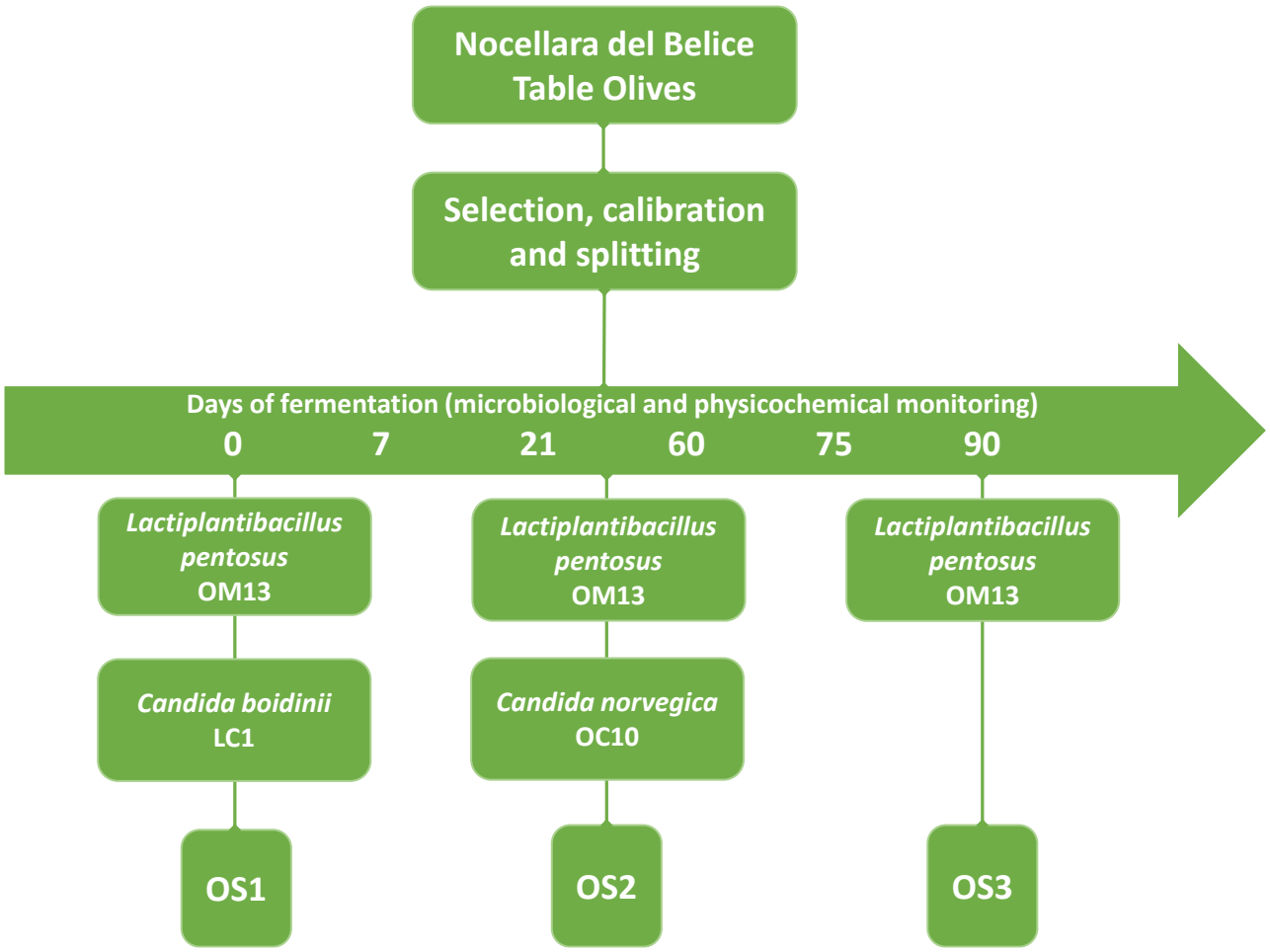
Physical parameters evaluated on green split Nocellara del Belice table olives after 90 days.

Trials	Drupe color			Pulp hardness (kg/cm^2)
	L	a*	b*	
OS1	53.20 ± 2.55[a]	-2.36 ± 1.34[b]	36.11 ± 1.15[ab]	11.61 ± 1.65[a]
OS2	53.19 ± 2.11[a]	-2.25 ± 1.39[b]	36.94 ± 1.42[a]	12.99 ± 1.39[a]
OS3	51.20 ± 2.10[a]	-0.94 ± 0.37[a]	33.17 ± 0.99[b]	5.84 ± 0.25[b]
Statistical significance	n.s.	*	*	*

Result indicate mean value (± standard deviation) of 30 olives.

According to Tukey's test, data in the same column followed by the same letter are not significantly different.. P value: *, $P < 0.05$; n.s., not significant.

Abbreviations: OS1, *Lactiplantibacillus pentosus* OM13 + *Candida boidinii* LC1; OS2, *L. pentosus* OM13 + *Candida norvegica* OC10; OS3, *L. pentosus* OM13 (control).



444

445

446 **Fig. 1.** Experimental design for the production of green split table olives. Abbreviations: OS1 and

447 OS2, codes refer to experimental treatments; OS3, code refers to control treatment.

448

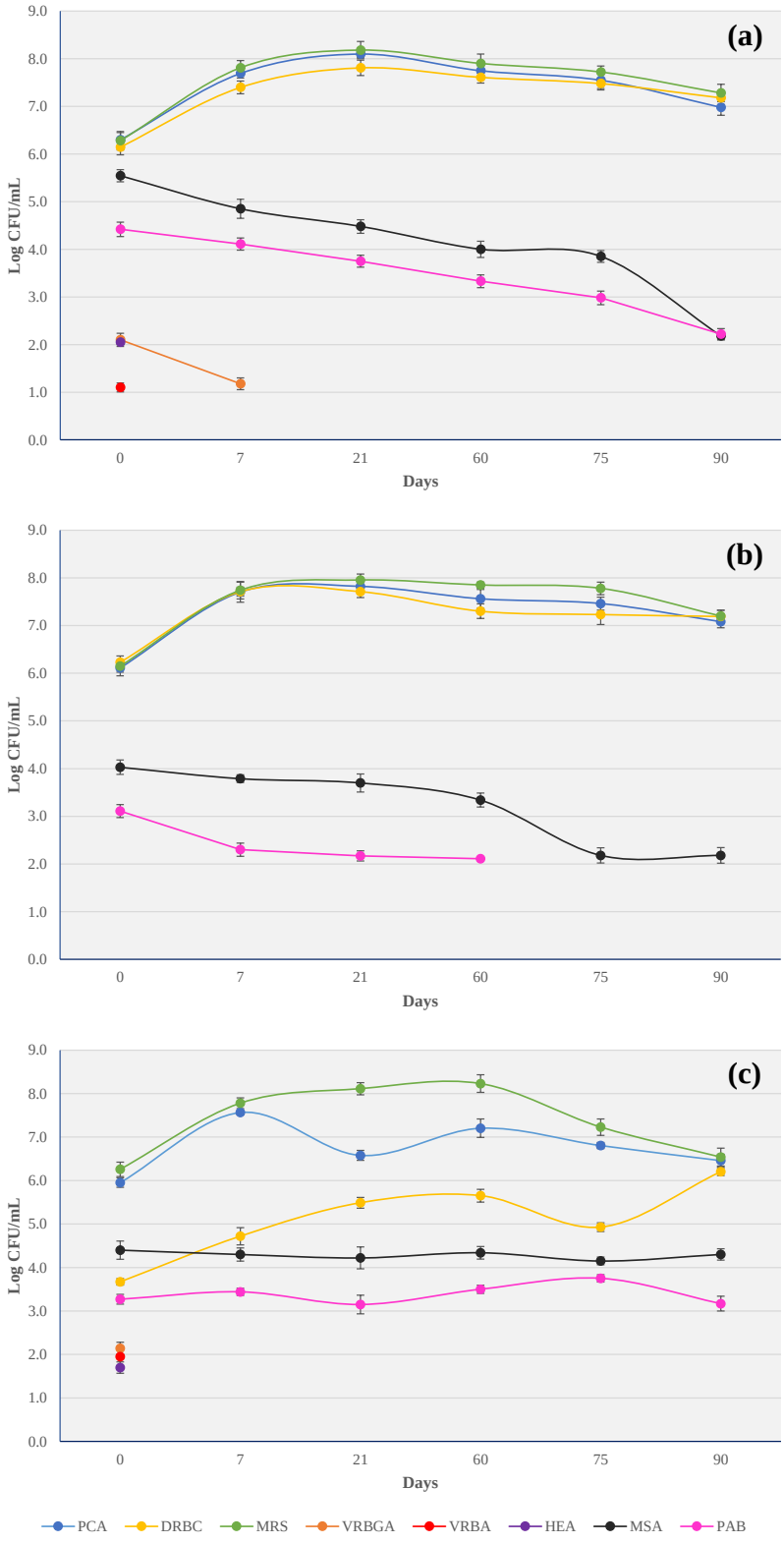


Fig. 2. Evolution of the microbial groups monitored during the production process of green split Nocellara del Belice table olives. Trials: **(a)** OS1, *Lactiplantibacillus pentosus* OM13 + *Candida boidinii* LC1; **(b)** OS2, *L. pentosus* OM13 + *Candida norvegica* OC10; **(c)** OS3, *L. pentosus* OM13 (control).

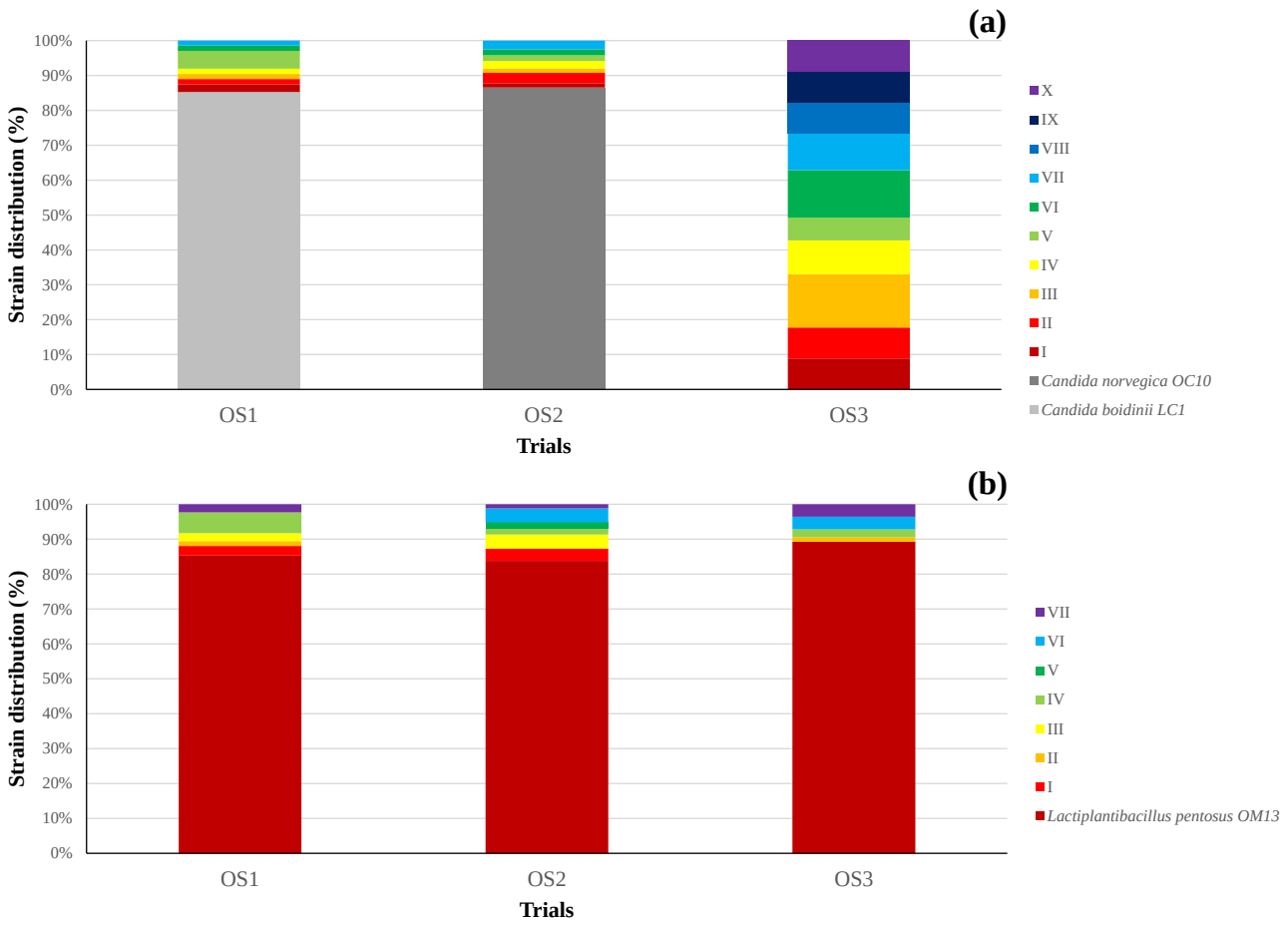
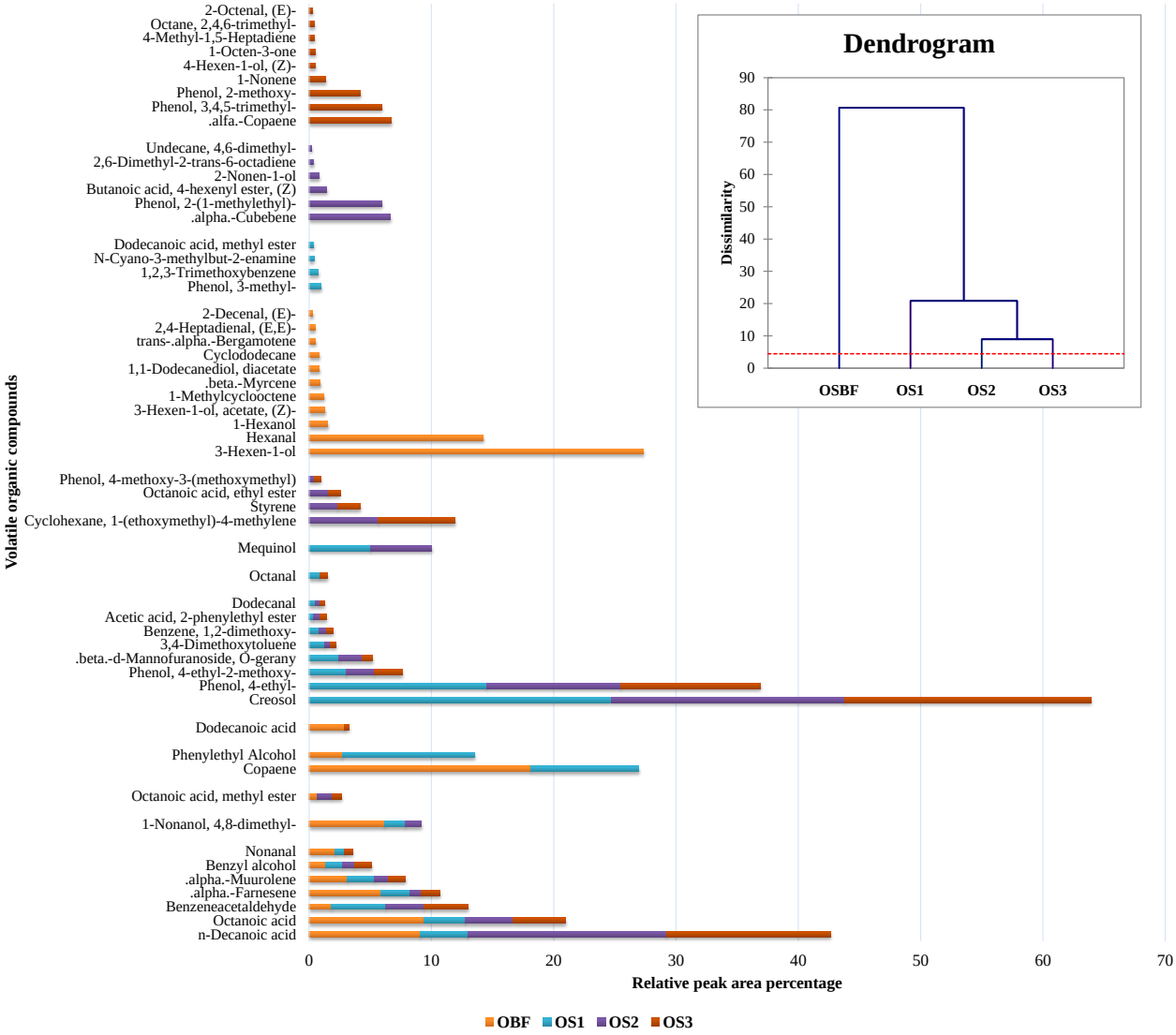


Fig. 3. Distribution of yeast strains (a) and lactic acid bacteria (b) during the fermentation of split Nocellara del Belice green table olives. The different RAPD profiles are represented by different coloured histograms (I-X).

Abbreviations: OS1, *Lactiplantibacillus pentosus* OM13 + *Candida boidinii* LC1; OS2, *L. pentosus* OM13 + *Candida norvegica* OC10; OS3, *L. pentosus* OM13 (control).



461

462 **Fig. 4.** Distribution and clustering of volatile organic compounds detected in Nocellara del Belice
463 split green table olives before the fermentation process (OBF) and after 90 days (OS1, OS2, OS3).
464 Abbreviations: OBF, olives before fermentation; OS1, *Lactiplantibacillus pentosus* OM13 + *Candida*
465 *boidinii* LC1; OS2, *L. pentosus* OM13 + *Candida norvegica* OC10; OS3, *L. pentosus* OM13 (control).

466

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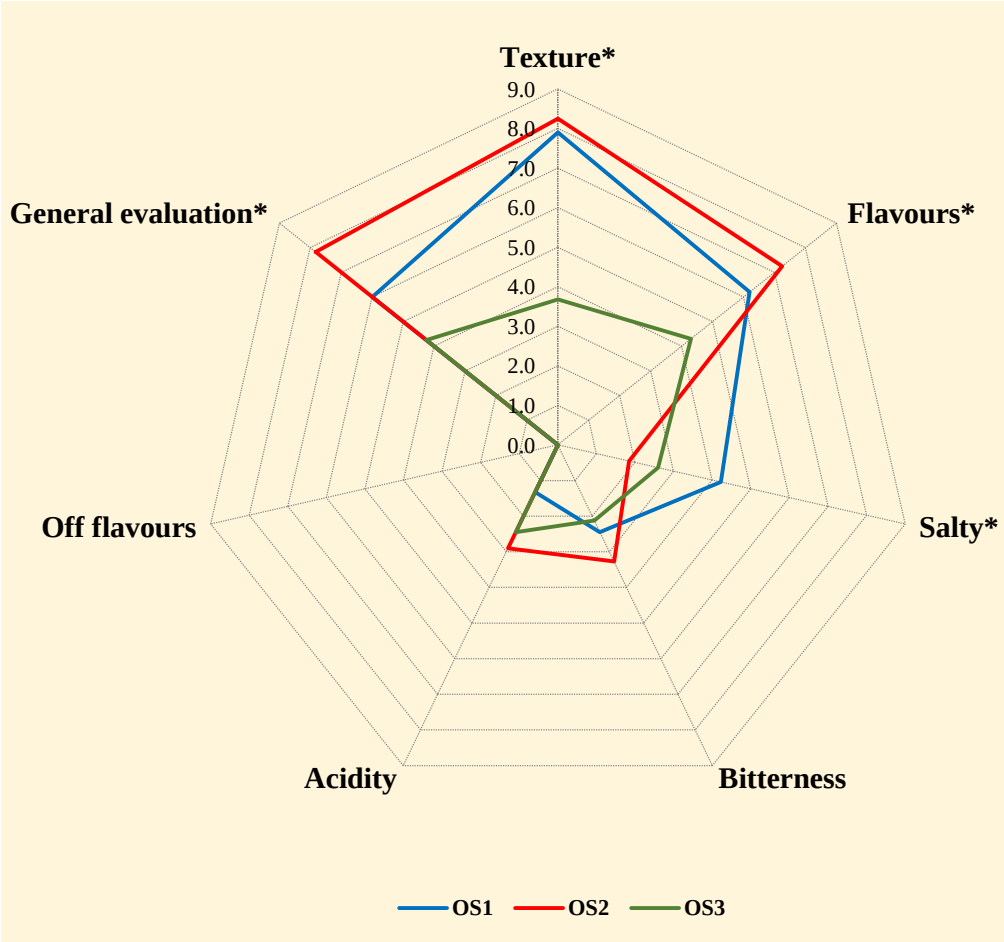

Fig. 5. Sensory profiles of green split table olives Nocellara del Belice at 90 days of fermentation. Abbreviations: OS1, *Lactiplantibacillus pentosus* OM13 + *Candida boidinii* LC1; OS2, *L. pentosus* OM13 + *Candida norvegica* OC10; OS3, *L. pentosus* OM13 (control).

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CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.