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Selection of starter cultures from olive skin to improve the quality of fermented table olives

Paola Foti [a], Lara Signorello [b], Maria Gullo [b,*], Mattia P. Arena [b], Nicolina Timpanaro [a], Cinzia Benincasa [c], Innocenzo Muzzalupo [d], Flora V. Romeo [a,**]

[a] Council for Agricultural Research and Economics (CREA), Research Centre for Olive, Fruit and Citrus Crops, Corso Savoia 190, 95024, Acireale (CT), Italy
[b] Department of Life Sciences, University of Modena and Reggio Emilia, Via Amendola, 2, 42122, Reggio Emilia, Italy
[c] Council for Agricultural Research and Economics (CREA), Research Centre for Olive, Fruit and Citrus Crops, Via Settimio Severo 83, 87036, Rende (CS), Italy
[d] Council for Agricultural Research and Economics (CREA), Research Centre for Forestry and Wood, Via Settimio Severo 83, 87036, Rende (CS), Italy

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ABSTRACT

The fermentation of olives, driven by indigenous microorganisms, can yield products of varying quality. This study evaluated the effectiveness of native starter cultures from the olive carposphere by fermenting Nocellara Messinese ripe olives in a 7 % (w/v) salt brine by using two Lactic Acid Bacteria (LAB) and one yeast strain in single and mixed combinations. The LAB isolated from the carposphere were subjected to phenotypic and molecular analyses for identification, and then deposited at Unimore Microbial Culture Collection UMCC). The fermentation was monitored through microbiological analyses of the brine samples along with physicochemical analyses of the olive pulp at the beginning and end of fermentation, and sensory analysis of final products was performed. All the inocula reduced the pH, with the indigenous selected LAB, achieving safe level (pH < 4.5) the fastest, while the uninoculated batch showed an increase in pH after 35 days of fermentation. Inoculated samples showed better total coliforms load reduction due to pronounced acidification and higher phenolic content in the brine. The findings suggest that using indigenous selected microbial starters leads to safe and effective productions, enhancing the quality of fermented table olives.

1. Introduction

Table olives (*Olea europaea* L.) represent the most widely consumed fermented food in the Mediterranean area [1]. Nowadays, table olives are consumed also for their high content of bioactive and health-beneficial compounds [2], such as phenols [3], fibres, and vitamins [4]. There is an increasing demand for foods with optimal nutritional characteristics, functional features and reduced synthetic additives [5]. Olive fermentation is generally conducted by indigenous microorganisms, whose activity depends on intrinsic and extrinsic factors resulting in a final variable quality product. In table olives produced by natural fermentation, the debittering process is performed exclusively by microorganisms and enzymes naturally occurring in the fermentation environment [6], but it is affected by the olive cultivar and physico-chemical conditions [7]. Indigenous fermentation has several disadvantages, such as the potential growth of pathogenic microorganisms and the consequence of undesirable and unpredictable final products [8]. To shorten and standardise the debittering phase of naturally fermented table olives, the use of selected lactic acid bacteria (LAB) starter cultures results in the improvement of the processes. In fact, the lowering of pH below safe levels (below 4.5) due to the rapid production of lactic acid, and also the acceleration of the debittering time due to LAB beta-glucosidase activity, positively affect the fermentation processes [9]. Indeed, the main biotechnological innovation for table olive fermentation is the use of selected starter cultures [10]. Starter cultures of LAB may consist of a single strain, or multiple strains, previously selected on the basis of specific technological characteristics, including organic acid production, salt tolerance, growth temperature range, ability to hydrolyse phenolic compounds, and bacteriocin production [11,12]. Among LAB, *Lactiplantibacillus plantarum* strains are widely

* Corresponding author.
** Corresponding author.
E-mail addresses: paola.foti@crea.gov.it (P. Foti), lara.signorello@unimore.it (L. Signorello), maria.gullo@unimore.it (M. Gullo), mattiapia.arena@unimore.it (M.P. Arena), nicolina.timpanaro@crea.gov.it (N. Timpanaro), cinzia.benincasa@crea.gov.it (C. Benincasa), innocenzo.muzzalupo@crea.gov.it (I. Muzzalupo), floravaleria.romeo@crea.gov.it (F.V. Romeo).

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used as starter cultures for vegetables fermentation and play a main role in table olives processes due to their high adaptability, their tolerance to stress conditions in brine, their ability to grow and cooperate with indigenous yeasts until the end of the fermentation and their ability to grow in presence of inhibiting phenolic compounds [11,13]. In olive fermentation, also the role of yeasts has been assessed. Yeasts can promote LAB growth and also be potential starters for their ability to improve the sensory profile of the product [14–16] and its antioxidant properties [17], although the use of yeasts as starters for olive fermentation is still at research level [18,19]. Among yeasts, *Wickerhamomyces anomalus,* which is one of the most effective biocontrol yeasts against fungal pathogens, has been previously detected in green and black table olives [20,21]. It has been recognised that naturally present strains in matrices can exhibit greater survival under different stress conditions than their commercial equivalents [22].

In the present study, a carpological analysis on black olives was conducted. Then, fermentations using two LAB and one yeast strain in single and mixed combinations were implemented and monitored to assess the quali-quantitative differences in the fermentation process on Nocellara Messinese natural black olives. Therefore, the aim of the study was the selection of new starter cultures for table olive industry, in order to improve the quality of final products, while reducing debittering times.

2. Materials and methods

2.1. Olive sampling and carpological analysis

During the 2021/2022 olive harvesting year, at the end of October, around 100 kg of pigmented olives of the Nocellara Messinese variety were kindly supplied by the farm 'Tenuta Vasadonna', located in Motta Sant'Anastasia in the province of Catania, Sicily.

The olives were selected, washed, graded, and subjected to carpological characterisation: determination of fruit and respective stone weight (g), diameter and length of the drupe (mm). Furthermore, 100 drupes were randomly sampled for the determination of maturation index (MI) according to Uceda and Frías [23].

2.2. Olive processing

The fermentation of Nocellara Messinese pigmented olives (MI = 4.0), was conducted in brine (1:1 ratio) with 7 % NaCl according to Benincasa et al. [24] into 5 L glass containers, with 1 % (v/v) inoculation LAB and yeasts according to the following trials. The starter cultures were inoculated to a final cell density of 8 Log CFU/mL for *L. plantarum* and 7 Log CFU/mL for *W. anomalus* directly after brining.

Strains DSM 20205 and DSM 6766 were grown according to the manufacturer's procedures (Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH, Germany). Brine samples were collected weekly until the end of fermentation for a period of 40 days and subjected to subsequent analyses.

2.3. LAB isolation and identification

The LAB used in the experimental design, at the paragraph 2.2, thesis C, were isolated from Carolea olive skin in a previous study, in which strain C showed the best technological characteristics (production of organic acids, olive debittering and sensory characteristics) [25]. The colonies, recovered on MRS agar (Biolife, Italy) plate, supplemented with cycloheximide (2 mg/mL, Oxoid, Italy) were randomly selected, purified, checked for Gram reaction and catalase activity and microscopically examined before storing in liquid culture using 30 % (v/v) glycerol (PanReac, Spain) at −80 °C. LAB strains were biochemically tested by API 50 CHL Medium kit (BioMerieux, France) and identified at species level with Apiweb™ software (BioMerieux, France).

Genomic DNAs (gDNA) was extracted by using DNeasy® powerfood® microbial kit (QIAGEN), following the manufacturer's instructions. The extraction was conducted starting from 1.8 mL overnight culture, grown in MRS broth, at 30 °C. gDNA was checked by 1.5 % (w/v) gel agarose in 0.5X TBE buffer and quantified by spectrophotometric measure (NanoDrop ND-1000). Band sizes were determined using a 100 bp DNA ladder (GeneRulerTM 100 bp plus DNA Ladder, Thermo Fisher Scientific).

gDNA was used as a template to amplify and sequence 16S rRNA gene. PCR analysis was performed according to Sato et al. [26]. The PCR primers used were: 27F (5′-CTGGGATCCATTTACTCGAGAGTTTGA TCCTGGCTCAG-3′), 1490R (5′-GGTTCCCCTAAGCTTACCTTGTTAC GACTTC-3′). The PCR mixture was 10 μL of 10X reaction buffer, 0.25 μL (5.0 U) of Taq DNA polymerase, 4.0 μL of dNTP (2.5 mM each), 1.0 μL of each primer (10 μM), 1.0 μL of DNA template (5 ng/μL) in a final reaction volume of 100 μL. PCR conditions were: 95 °C for 5 min, 30 cycles of 94 °C for 1 min, 58 °C for 2.5 min, 72 °C for 2.5 min, and final extension at 72 °C for 5 min.

Then, PCR amplicons of the 16S rRNA gene were sequenced using the following primers: 27F (5′-CTGGGATCCATTTACTCGAGAGTTTGA TCCTGGCTCAG-3′), 1490R (5′-GGTTCCCCTAAGCTTACCTTGTTACGAC TTC-3′) [27], 16SF (5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCWGCAG-3′), 16SR (5′-GTCTCGTGGGCTCGGAGATG-TG TATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) [26,28]. Sequencing was performed through DNA Sanger dideoxy sequencing process by Bio-Fab Research (Rome, Italy). Sequencing data were analysed using the SnapGene 7.20 (GSL Biotech, available at https://www.snapgene.com). 16S rRNA sequence was matched using the basic local alignment search tool NCBI BLAST (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the most related DNA sequence relatives in the NCBI nucleotide sequence database. 16S rRNA gene sequence was deposited at GenBank under the accession number PQ518644.

Subsequently, the full-length 16S rDNA sequence after alignment was used for phylogenomic analysis by the UPGMA method [29] using MEGA software (version 11.0.13) [30]. The analysis involved 99 full-length 16S rDNA sequences. The distances were computed using the Maximum Composite Likelihood method [31]. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

2.4. Microbiological analyses

The samples were microbiologically analysed up to 40 days of fermentation using the following selective media and conditions: de Man Rogosa and Sharpe Agar (MRSA, Oxoid, Italy) added with 50 mg/L Nystatin [32] for LAB stored at 32 °C for 48 h under anaerobic conditions. Plate Count Agar (PCA, Oxoid, Italy) for aerobic mesophilic bacteria incubated at 25 °C for 48 h. Sabouraud Chloramphenicol agar (SAB, Bio-Rad, Italy) for yeasts and moulds at 25 °C for 48 h. Chromogenic coliform Agar Base (CCA, Bibby Scharlau, Italy) for coliform bacteria at 37 °C for 24 h; Mannitol Salt Agar (MSA, Oxoid, Italy) for coagulase-positive staphylococci at 32 °C for 72 h. The brine analyses were conducted in triplicate. The results were expressed as Log CFU/mL of brine.

2.5. Chemical and physical analysis

2.5.1. Brine samples

The acidification kinetics of the brine was measured in the samples by analysing the pH value. The pH was detected by using a MettlerDL25 pHmeter (Mettler-Toledo International Inc., USA) provided with buffer solutions for calibration at 4.0 and 7.0 pH values (Crison, Barcelona).

The olive phenols in brine samples were monitored through HPLC analyses by directly injecting the filtered brine samples (0.45 μm filters, Millipore, USA) in the chromatographic HPLC system. The system consisted of a liquid chromatography Waters Alliance 2695 HPLC equipped with a Waters 996 photodiode array detector (PDA) set at 280 nm and with Waters Empower software (Waters Corporation, USA). The column

was a Luna C18 (250 mm × 4.6 mm i.d., 5 μm, 100 Å; Phenomenex, USA) maintained at 40 °C. An injection volume of 20 μL and a flow of 1 mL/min were used. Chromatographic separation was achieved by elution gradient using an initial composition of 95 % of A solution (water acidified with 2 % acetic acid) and 5 % of B solution (methanol). Solvents were HPLC grade (Merck KGaA, Germany). The B solution increased to 30 % in 15 min and to 70 % in 25 min and then, after 2 min in isocratic, the mobile phase was set at the initial conditions for 8 min. The internal standard (I.S.) of 5 mM pure gallic acid (Fluka, Switzerland) was used to quantify the phenolic compounds. All the analyses were carried out in triplicate for each sample. Phenolic compounds were identified by injecting the pure standards (Extrasynthése, France) of hydroxytyrosol (TyrOH), tyrosol (Tyr), oleuropein (Ole) and verbascoside (Verb), and by means of their retention time and UV–Vis spectra [33]. All the analyses were performed in triplicate.

2.5.2. Olive pulp samples

Phenols of olive pulp were determined by following extraction procedures optimised at CREA laboratory [34]. Briefly, 1 g of homogenised olive drupes was treated with 10 mL of an hydroalcoholic solution, consisted of water/methanol (v/v 20:80), kept under shaking in an ultrasonic bath (Fisher Scientific, Milan, Italy) in darkness for 20 min. Centrifugation at 5000 rpm for 25 min at 8 °C allowed the recovery of the supernatant which, after being filtered through a 0.45-μm PVDF filter (Merk, Darmstadt, Germany), was analysed by mean of a MSD Sciex Applied Biosystem API 4000 Q-Trap mass spectrometer in negative ion mode using multiple reaction monitoring (MRM): for each compound of interest, the transition of the deprotonated molecular ion was scanned on the first quadrupole and its main fragments on the third one. The analytes were separated on an Eclipse XDB-C8-A HPLC column (5 μm particle size, 150 mm length and 4.6 mm i.d.) at a low rate of 250 μL/min. A binary mobile phase made up of 0.1 % aqueous formic acid (A) and methanol (B) was gradient programmed and the total elution time was 20 min per injection. Quantitative analyses were performed by external calibration curves, at concentrations ranging between 100 and 2000 μg/mL, built using a least-squares linear-regression analysis. The analytical standards were purchased from Sigma-Aldrich (Riedel-de Haën, Laborchemikalien, Seelze, Germany) and Extrasynthése (Genay, France).

For sugar analysis, approximately 30 g of drupes were dried in a static oven at 60 °C until no change in weight was recorded and extracted with distilled water using an ultrasonic bath (15 min), followed by centrifugation (10 min) and filtration (PTFE filter 0.2 μm, 25 mm, Whatman) [35]. The filtered supernatant solution was analysed by LC–MS in positive-ion mode using multiple reaction monitoring (MRM): for each analyte, the transition of the deprotonated molecular ion $[Sugar\text{-}H]^+$ was scanned on the first quadrupole and its main fragments on the third one $[Sugar\text{-}Cs]^+$. Few millimolar solution of CsCl salt was required to generate stable Cs adducts and enhanced sensitivity. 10 μL of analytes, mannose, fructose, glucose and galactose, were separated on a Chromegabond carbohydrate column [(5 μm particle size, 15 cm length and 2.1 mm i.d. (PerkinElmer, West Berlin, NJ, USA)] at a flow rate of 300 mL/min. The mobile phase consisted of acetonitrile (A) and CsCl H_2O 54 μM (B). Quantitative analyses were achieved by external calibration curves, at concentrations ranging between 0.625 and 10 μg/mL, built using a least-squares linear regression analysis with correlation coefficients between 0.9996 and 0.9999 (Fig. S1, Supplementary Material). Sugar standards of mannose, fructose, glucose and galactose were purchased by Sigma-Aldrich (Riedel-de Haën, Laborchemikalien, Seelze, Germany).

2.6. Sensory analysis

Sensory analyses were performed at the CREA sensory analysis laboratory (Acireale, Italy), devised in accordance with ISO 8589:2007/Amd 1:2014 (Sensory analysis - General guidelines for the design of test rooms Amendment 1) standard [36] and equipped with specific software for sensory data acquisition (Smart Sensory box, Smart Sensory Solutions S.r.l., Sassari, Italy).

The Sensory profile technique according to the standard ISO 13299:2016 was used [37].

The sensory evaluation of the tested olives was carried out following the guidelines of the International Olive Council (COI/OT/MO No 1/Rev.June 3, 2021) [38]. The sensory analysis was carried out by a group of 9 experienced tasters. The judges were trained [39] for identification of abnormal fermentation (putrid, butyric, zapateria), for gustatory attributes (saltiness, bitterness, acidity), for kinaesthetic or texture attributes (hardness, fibrousness, crunchiness), and 'other defects' of table olives (mustiness, rancidness, cooking effect, metallic taste, etc.). As indicated by the COI, the scale used measures 10 cm in length and the intensity varies from 1 to 11. Standard tasting glasses, containing 3 olives with the brining liquid, were evaluated by each judge. Samples were presented to the judges at room temperature and all samples were labelled with three-digit codes. The codes were automatically produced by the smart sensory box. Two samples at each session were tasted (one sample and one replicate in a total of 5 sessions).

2.7. Statistical analysis

Statistical analysis of the results was performed using one-way analysis of variance (ANOVA), and Tukey's HSD post-hoc test for separation of the means (significance level $p \leq 0.05$), and 95 % Confidence Intervals (CI) using the statistical software IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, N.Y., USA).

3. Results and discussion

3.1. Carpological characterisation

The carpological characteristics of Nocellara Messinese black olives are shown in Table 2. The olives were treated at fully ripe state and the drupe sizes were lower than those described by Timpanaro et al. [40], compared to the same cultivar harvested at green state during 2020 in Sicily. In this study, the flash/pit ratio of pigmented olives was 5.46, less than 6.4 reported by the authors [40], but still suitable for table olives production. The weight was also much lower (6.04 g versus 9.14 g), but this could be due to the loss of water of the fruits during the ripening process and to the different producing farm.

3.2. Bacterial strain identification

One of the strains (named OC1) isolated from olive skin, was selected and analysed for its carbohydrate fermentation patterns by using a biochemical API 50 CHL system kit. The results of all the wells were then analysed through the Apiweb™ software. OC1 isolate was able to completely ferment 25 out of 49 tested substrates, as shown in Table 3.

The biochemical profile corresponded to *L. plantarum* with an identification rate of 99.9 %. *L. plantarum* is frequently isolated from fresh and fermented plants, due to its broad habitat range [41]. The species is typically highly abundant in olive fermentations [4,7,11,13], as confirmed by several authors, while there is very little research on the isolation of these bacteria from olive skin.

Table 1
Experimental fermentation of brined olives.

Trial	Treatment (inoculum)
A	*Lactiplantibacillus plantarum* (DSM 20205)
B	*Wickerhamomyces anomalus* (DSM 6766)
C	*Lactiplantibacillus plantarum* strain (OC1)
D	Mix of strains of A, B, and C trials
E	Un-inoculated spontaneously fermented control samples

Table 2
Carpological characteristics of olive drupes.

	Flesh diameter (mm)	Flesh length (mm)	Fruit weight (g)	Flesh weight (g)	Stone weight (g)	flesh/pit
Nocellara Messinese	19.73 ± 1.82	25.43 ± 2.05	6.04 ± 1.20	5.09 ± 1.09	0.95 ± 0.19	5.46

Data are expressed as means ± standard deviations.

Table 3
Biochemical profile of the API 50 CH Kit of the OC1 isolated strain.

Well n.	Biochemical test	Result	Well n.	Biochemical test	Result
0	Control	-	25	Esculine	+
1	Glycerol	-	26	Salicin	+
2	Erythritol	-	27	Cellobiose	+
3	D-Arabinose	-	28	Maltose	+
4	L-Arabinose	+	29	Lactose	+
5	Ribose	+	30	Melibiose	+
6	D-Xylose	-	31	Sucrose	+
7	L-Xylose	-	32	Trehalose	+
8	Adonithol	-	33	Inulin	-
9	Methyl xyloside	-	34	Melizitose	+
10	Galactose	+	35	D-Raffinose	+
11	D-Glucose	+	36	Starch	-
12	D-Fructose	+	37	Glycogen	-
13	D-Mannose	+	38	Xylitol	-
14	Sorbose	-	39	Gentibiose	+
15	Rhamnose	-	40	Turanose	+
16	Dulcitol	-	41	Lyxose	-
17	Inositol	-	42	Tagatose	-
18	Mannitol	+	43	D-Fucose	-
19	Sorbitol	+	44	L-Fucose	-
20	Methyl-D-mannoside	+	45	D-Arabitol	-
21	Methyl-D-glucoside	-	46	L-Arabitol	-
22	Methyl-D-glucosamine	+	47	Gluconate	+
23	Amygdalin	+	48	2, Keto-gluconate	-
24	Arbutin	+	49	5, Keto-gluconate	-

Patrial-length 16S rRNA gene sequencing and analysis (1418 bp) revealed more than 99 % similarity with the 16S rRNA of *L. plantarum* sequences retrieved from GenBank data library. A phylogenetic analysis indicated that OC1 was closely related to the strain *L. plantarum* P9, *L. plantarum* beLP1, and *L. plantarum* KCKM 0597 (Fig. 1). According to API 50 assays and 16S rRNA analysis the strain was assigned to the species *L. plantarum*. Strain OC1 was deposited at UMCC (Unimore Microbial Culture Collection) under the accession code UMCC 3073.

3.3. Microbiological analyses of brine samples

All the fermented olive samples were analysed for their microbial composition, as highlighted in Table 4. LAB began to show a quantitative difference among the trials only at 12 days of fermentation, when C and D trials reached the highest concentration, up to the end of fermentation. Both trials contained *L. plantarum* OC1, as a single strain (C) or in a mixture (D).

Instead, trial A, produced by inoculating *L. plantarum* (DSM 20205) showed 1–2 logarithmic units lower compared to OC1 samples. In particular, trial A showed an increase up to 26 days of fermentation and then a decrease at the end of sampling, while trials C and D showed a constant count to the end of fermentation time. As underlined by Vaccalluzzo et al. [11] the LAB's behaviour is strain-dependent regarding the stress factors of the growth conditions. In the present study, the inoculation of the OC1 strain led to a higher count of LAB with respect to the other trials. Although this does not mean that the LAB count is entirely due to OC1 cells, it indicates that OC1 drove the fermentation.

Randazzo et al. [7] highlighted that *L. plantarum* can properly drive the fermentation processes even without dominating in brines. Yeasts and moulds count reached the plateau of about 7.6 Log CFU/mL at 19 days (data not shown) and remained quite constant for another week. However, at the last two sampling times, sample C reached the lowest values, as if OC1 strain had a containing effect on yeasts and moulds growth.

This behaviour might be possible due to antifungal compounds produced by LAB, especially low molecular mass metabolites, organic acids and in particular propionic acid [42]. No effect was instead shown by OC1 strain versus the total mesophilic aerobic count, which was high in all the trials, showing the lowest values in A and B samples at 40 days of fermentation. Coagulase-positive staphylococci were present at the beginning of fermentation in all the samples but disappeared along fermentation time, probably due to the acidification of the products. However, from day 26 their growth increased, reaching the lowest value at 40 days in A, B and C samples. Total coliforms were always found in all brine samples and started to decrease at day 33 (data not shown), when C and D samples reached the lowest values and then C at the end of fermentation reached a safety count. Randazzo et al. [7] showed that *Enterobacteriaceae* population was reduced faster by using *L. pentosus* than *L. plantarum*, but in the present study OC1 strain significantly reduced coliforms. Unlike these results, the use of starters for Bencresciuto et al. [19] did not improve the LABs' growth nor prevent the growth of *Enterobacteriaceae* and moulds. The reasons for these differences could depend on the different pH of the brine and the diversity of strains used. The use of OC1 strain can assure higher safety attributes of the fermented olives with respect to the other used starters. Finally, a positive effect of *W. anomalus* (B), inoculated alone or mixed with the two *L. plantarum* (D), was observed only on the 40th day versus the staphylococci count.

3.4. Chemical analyses

3.4.1. Brine samples

The brine of the different trials was analysed for their pH value at each sampling time and the results are shown in Fig. 2. The pH values decreased in all samples and clearly started to rise again at 40 days in the control sample (E). Statistical differences among the samples were observed ($p \leq 0.05$) starting from 19 days to the end. C samples reached the lowest pH values (4.59) different from the others at day 19, then C and D at days 26 (4.36 and 4.48, respectively) and 33 (4.21 and 4.33, respectively), while A, C and D at day 40 (4.38, 4.24, 4.39, respectively). At the last two sampling time, all the inoculated samples were different from the control one, reaching a safe pH value. A LAB inoculum usually maintains a lower pH value than the control samples due to a faster acidification of brine [43,44].

In Table 5, the results of individual phenols from brine samples are shown. Hydroxytyrosol (HT) content increased up to the end of fermentation in all trials. HT was significantly higher in A and C trials at 12 and 19 days. This result was expected since the A and C samples were inoculated with two different *L. plantarum* strains, which are known to possess β-glucosidase activity. The oleuropein (OLE) was never detected in all the samples at any monitoring time. It is well-known that β-glucosidase enzyme is related to oleuropein hydrolysis and that *L. plantarum* strains are used as starter culture mainly for debittering olives [4,9]. Moreover, OLE decreases as ripening progresses and in this study black ripe olives were used. Tufariello et al. [18] found OLE undetectable in black fermented olives of different cultivars with the only exception of Cellina di Nardò drupes. Romeo et al. [44] found OLE in brine of black fermented olives at 30 days but non at 60 days of fermentation. Tyrosol (TY) was detected starting from 19 days when was

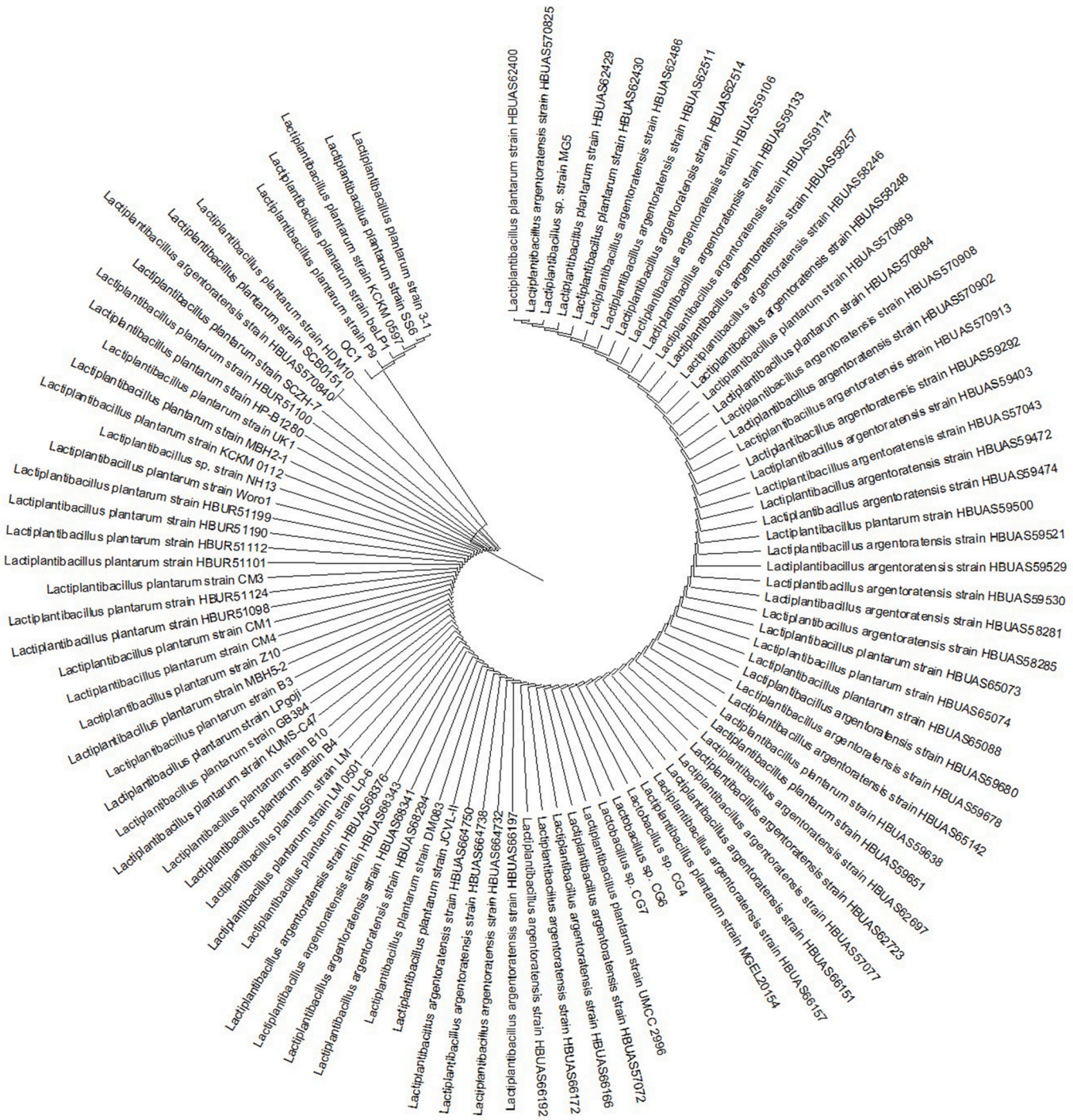

Fig. 1. Phylogenetic tree showing relationship of OC1 strain with other *L. plantarum* strains.

higher in A, B and C inoculated samples. Then, reached the highest values in C and D at 33 days and in A, C and D at the end of fermentation, i.e. in all the samples inoculated with *L. platarum* strains. It is known that the fermentation process can increase the total polar phenols release from the olives, especially by using selected starters [45]. Regarding the verbascoside (VB), it was detected starting from 19 days as tyrosol and highlighted significant differences only at this sampling time, by reaching the highest values in A and C samples. The increasing trend of HT, TY and VB during fermentation were in accordance with other researchers [4,24,33].

3.4.2. Olive pulp samples

The difference in total phenolic content (TPC) between fresh olives pulp and table olives was statistically significant. Olives, as expected, showed an important loss in TPC during fermentation due to the diffusion of these compounds to the brine but, TPC was found to be high in fermented samples too (Table 6). More specifically, at the start, TPC of fresh olives pulp was 10603 mg/kg; this value was consistent with the data reported in the literature relating to the TPC of fresh olives belonging to Nocellara Messinese cultivar [24,46]. Subsequently, the starter cultures acted diversely leading to different but still satisfactory results in terms of TPC and individual phenols [47,48].

The fermentation process that showed the lowest reduction in terms

Table 4
Log CFU/mL counts of microbial populations in olive brines.

Trial	Time (d)	Lactic Acid Bacteria	Yeasts & Moulds	Total mesophilic aerobic count	Coagulase-positive Staphylococci	Total Coliforms
A	5	4.47 ± 0.41	5.25 ± 0.24	5.28 ± 0.20	4.54 ± 0.51a	2.85 ± 0.15abc
B		4.60 ± 0.28	4.66 ± 0.25	5.78 ± 0.78	3.31 ± 0.27b	2.00 ± 0.05c
C		5.02 ± 0.30	5.28 ± 0.16	5.59 ± 0.47	3.67 ± 0.04 ab	3.66 ± 1.11 ab
D		4.63 ± 0.39	4.78 ± 0.60	4.75 ± 0.40	3.50 ± 0.68b	2.59 ± 0.13bc
E		4.89 ± 0.34	5.01 ± 0.49	5.04 ± 0.34	4.41 ± 0.15a	4.04 ± 0.78a
95 % CI		Upper 4.90	Upper 5.20	Upper 5.55	Upper 4.18	Upper 3.46
		Lower 4.55	Lower 4.80	Lower 5.02	Lower 3.59	Lower 2.59
Sig.		n.s.	n.s.	n.s.	**	**
A	12	6.07 ± 0.59bc	6.99 ± 0.63 ab	6.86 ± 0.53a	0.00 ± 0.00	5.34 ± 1.10
B		6.09 ± 0.07bc	6.36 ± 0.15 ab	6.38 ± 0.16 ab	0.00 ± 0.00	5.27 ± 0.61
C		6.89 ± 0.16a	7.30 ± 0.14a	7.11 ± 0.22a	0.00 ± 0.00	6.23 ± 0.04
D		6.23 ± 0.17 ab	6.80 ± 0.72 ab	6.60 ± 0.60 ab	0.00 ± 0.00	5.35 ± 1.12
E		5.47 ± 0.38c	6.06 ± 0.05b	5.80 ± 0.27b	0.00 ± 0.00	4.57 ± 0.41
95 % CI		Upper 6.40	Upper 6.98	Upper 6.82	Upper -	Upper 5.77
		Lower 5.89	Lower 6.42	Lower 6.28	Lower -	Lower 4.94
Sig.		**	**	**	n.s.	n.s.
A	26	6.85 ± 0.23b	7.08 ± 0.26b	7.01 ± 0.03b	6.13 ± 0.69a	6.58 ± 0.22a
B		6.82 ± 0.41b	7.22 ± 0.52 ab	6.53 ± 0.40c	5.99 ± 0.78a	6.10 ± 0.95 ab
C		7.80 ± 0.14a	7.80 ± 0.14a	7.76 ± 0.12a	4.35 ± 1.07b	5.31 ± 0.38b
D		7.73 ± 0.13a	7.29 ± 0.30 ab	7.82 ± 0.11a	5.66 ± 0.23 ab	5.90 ± 0.41 ab
E		5.43 ± 0.15c	7.10 ± 0.09b	7.49 ± 0.10a	6.62 ± 0.16a	6.33 ± 0.10 ab
95 % CI		Upper 7.35	Upper 7.48	Upper 7.57	Upper 6.22	Upper 6.33
		Lower 6.50	Lower 7.12	Lower 7.07	Lower 5.292	Lower 5.75
Sig.		**	*	**	**	*
A	40	5.45 ± 0.27c	5.00 ± 0.23 ab	5.45 ± 0.65c	2.66 ± 3.07b	4.08 ± 2.05 ab
B		6.04 ± 0.68bc	5.82 ± 0.86a	6.19 ± 0.62bc	1.41 ± 1.02b	3.78 ± 2.00 ab
C		7.29 ± 0.22 ab	4.20 ± 0.56b	7.09 ± 0.16 ab	2.55 ± 0.82b	1.28 ± 0.48b
D		7.53 ± 0.14a	6.12 ± 0.54a	7.31 ± 0.17a	3.54 ± 0.32 ab	4.33 ± 1.23 ab
E		3.03 ± 1.20d	5.60 ± 0.10a	6.99 ± 0.25 ab	6.66 ± 0.34a	6.75 ± 0.22a
95 % CI		Upper 6.70	Upper 5.74	Upper 6.98	Upper 4.45	Upper 5.10
		Lower 5.05	Lower 4.95	Lower 6.23	Lower 2.28	Lower 2.81
Sig.		**	**	**	**	**

Data are expressed as means ± standard deviations. Different letters indicate statistical differences within the same column for each sampling time for Tukey's test. CI= Confidence intervals. **Significance at $P \leq 0.01$; *significance at $P \leq 0.05$; n.s. not significant.

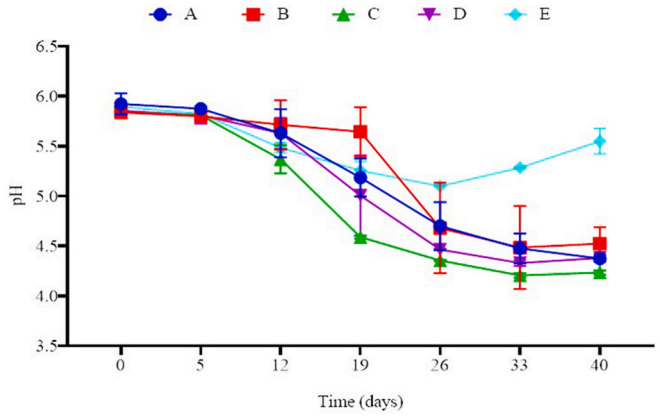

Fig. 2. Results of pH trend in olive brines at the different sampling time. Data are expressed as means and standard deviation bars (The legenda of the trials is included in Table 1).

of TPC in olives (8337 mg/kg) was the one in which only one yeast (B) was used [19,49]. It can be assumed that the use of *W. anomalus* (DSM 6766), positive for β-glucosidase activity, does not necessarily correlate with the ability to hydrolyse oleuropein. These observations are in agreement with those reported by other authors. In particular, Ruiz-Barba et al. [47] showed that the use of *W. anomalus* as a starter failed to reduce the total phenol or oleuropein content in Gordal, Manzanilla and Hojiblanca olive pulps compared with the corresponding un-inoculated control. On the other hand, Bencresciuto et al. [19] showed that final levels of oleuropein in Leccino table olives were not affected very much by the action of LAB starters. Natural fermentations (E) and fermentation driven by *L. plantarum* strain (C) produced olives with a TPC content decreased by a 40 % compared to its initial value (6196 and 6140 mg/kg, respectively); *L. plantarum* (A) and the mix of LAB and yeast (D) produced olives with a TPC content of 7181 and 7116 mg/kg, respectively, and corresponding to a TPC content decreased by a 30 % compared to the initial value. Individual phenols have a similar trend as TPC. Tyrosol, hydroxytyrosol, oleuropein, ligstroside and verbascoside were higher in olives treated with *W. anomalus* (B). Higher concentrations of verbascoside were registered in all theses with the exception of the control. An increase of verbascoside during the processing could be due to a conversion of hydroxytyrosol, as suggested by Sahan et al. [50]. Verbascoside content, also, varies greatly in olive naturally fermented as reported by Morelló et al. [51]. For individual phenols, vanillic acid and coumaric acid, the different starter cultures made no difference, in fact, statistical significance was only observed between fermented and non-fermented olives. Apigenin, lutein and lutein-4-O-glucoside values decreased with fermentation and statistical differences occurred in olives treated with starter cultures and natural ones. Rutin didn't show any significant difference among the trials.

As regards sugars, they are the main soluble components in olive tissues providing energy and carbon sources to microorganisms producing also secondary metabolites during fermentation. The initial content of soluble sugars, sum of mannose, fructose, glucose and galactose of fresh olive pulp was 11.25 g/100 g dry weight (DW). This value was significantly reduced during fermentation: after forty days of inoculation by the starter cultures, in fact, reduction rates of between 33 % and 66 % were recorded. Larger decreases, between 71 % and 79 %, have been reported in previous studies for four green olive cultivars typical of the Moroccan market [52].

In our study the greatest reduction of sugars was observed in trial D (3.77 g/100 g DW) and B (4.93 g/100 g DW). As hypothesised in several

Table 5
HPLC results of individual phenols (mg/L) in olive brines at different time of sampling.

Trial	Time (d)	Hydroxytyrosol	Tyrosol	Verbascoside
A	12	445.9 ± 82.2 ab	0.0 ± 0.0	0.0 ± 0.0
B		335.5 ± 4.9c	0.0 ± 0.0	0.0 ± 0.0
C		490.3 ± 50.0a	0.0 ± 0.0	0.0 ± 0.0
D		366.5 ± 23.6bc	0.0 ± 0.0	0.0 ± 0.0
E		345.9 ± 20.4c	0.0 ± 0.0	0.0 ± 0.0
95 % CI		Upper 432.5	Upper -	Upper -
		Lower 362.1	Lower -	Lower -
Sig.		**	n.s.	n.s.
A	19	959.1 ± 177.2 ab	31.4 ± 7.8 ab	34.2 ± 5.9a
B		766.1 ± 8.1c	25.8 ± 1.1 ab	23.6 ± 1.5b
C		1024.1 ± 37.1a	37.5 ± 4.4a	32.3 ± 4.4a
D		830.3 ± 39.7bc	23.7 ± 2.7c	22.1 ± 4.5b
E		790.3 ± 29.2bc	22.8 ± 8.4c	00.0 ± 0.0c
95 % CI		Upper 933.5	Upper 31.8	Upper 28.5
		Lower 814.5	Lower 24.7	Lower 16.4
Sig.		*	*	**
A	33	1509.1 ± 48.1	57.8 ± 20.8b	60.4 ± 19.1
B		1562.9 ± 60.5	49.4 ± 3.0b	57.7 ± 6.2
C		1645.0 ± 132.2	117.5 ± 31.8a	68.7 ± 4.9
D		1425.8 ± 263.8	87.5 ± 17.3 ab	50.0 ± 13.6
E		1620.5 ± 30.6	50.2 ± 2.7b	63.7 ± 2.5
95 % CI		Upper 1681.3	Upper 87.3	Upper 66.7
		Lower 1424.0	Lower 57.5	Lower 53.5
Sig.		n.s.	**	n.s.
A	40	2003.6 ± 272.2	88.5 ± 16.2 ab	97.6 ± 14.5
B		1978.0 ± 25.7	77.2 ± 7.0bc	95.6 ± 13.8
C		2055.2 ± 27.2	105.4 ± 0.8a	117.7 ± 6.5
D		2123.1 ± 350.9	94.2 ± 17.2 ab	91.8 ± 20.0
E		2110.7 ± 28.7	60.2 ± 6.5c	100.5 ± 9.7
95 % CI		Upper 2141.6	Upper 93.9	Upper 107.8
		Lower 1966.7	Lower 76.3	Lower 93.5
Sig.		n.s.	**	n.s.

Data are expressed as means ± standard deviations. Different letters indicate statistical differences within the same column for each sampling time for Tukey's test. CI= Confidence intervals. **Significance at P ≤ 0.01; *significance at P ≤ 0.05; n.s. not significant.

works, in co-inoculation fermentation there is a synergistic effect between the action of LAB and yeasts [52]. Interestingly, the lowest reduction in terms of TPC had occurred in olives. Glycosides present in olive pulp (including oleuropein) released, in fact, soluble sugars when hydrolysed.

The highest value of soluble sugars (6.88 g/100 g DW) was observed in un-inoculated fermented control samples. These results have pointed out that LAB and yeasts obviously led to a faster consumption of sugars effused from the olive fruits into the brines. On the contrary, the delay in the sugar consumption observed in fermentation could be attributed to the absence of LAB. All the values observed are listed in Table 6.

3.5. Sensory analysis

The ANOVA analysis (Table S1, Supplementary Material) indicated that the other defects, salty, bitter, acid, hardness and fibrousness descriptors were statistically significant. The sensory analysis of the samples showed that the bitterness score was significantly higher in control sample E compared to the other treatments (Fig. 3). This result highlights that all the inocula have positively influenced the kinetics of olive debittering in accordance with the findings of Lanza et al. [15]. Furthermore, the kinaesthetic descriptors showed low values, due to the presence of ripe olives which generally lose their hardness quickly. About this last descriptor, only the thesis inoculated with the *W. anomalus* yeast was affected but, it is commonly accepted that some yeasts, due to their enzymatic set (pectolytic enzymes), can influence the consistency of the drupes. Indeed, the yeasts contribute to the taste and aroma of the final product, but on the other hand, the CO_2 and enzymes produced by the yeasts can cause softening of the olives and spoilage of the final product in the early stages of fermentation [53].

Furthermore, sample D was statistically saltier than the other theses and sample B was different for being perceived with the greatest acidity.

4. Conclusions

In this study different starter cultures aimed at producing fermented table olives were assessed. Results highlighted the best reduction of the pathogenic load, thanks to a marked acidification and a higher content of phenols in brine, using a LAB strain previously isolated from olive drupe skin. In particular, the *L. plantarum* OC1 strain, was the most effective in containing the total coliform bacteria, yeasts and moulds load. It is therefore important to identify and characterise native starter cultures to optimise biotechnological applications in the fermentation process of table olives, paying attention to the technological characterisation of the strains, as well as to the sensory quality of the final product. Although sensory characteristics depend on several factors, in this study a significant contribution was given by the starter culture, not only in reducing the bitterness, but also in giving a characteristic flavour as well as helping to standardise the production. Since results of this

Table 6
LC-MS results of individual, total phenols (mg/kg) and soluble sugars (g/100 g dry matter) in olive pulp at the beginning and at the end of fermentation.

	Olive pulp	A t40	B t40	C t40	D t40	E t40	95 % CI		Sig.
	t0						Lower	Upper	
tyrosol	78.2 ± 6.9d	159.6 ± 7.4abc	173.7 ± 11.4a	144.4 ± 7.8bc	171.9 ± 14.1 ab	138.5 ± 12.7c	127.3	161.5	**
hydroxytyrosol	279.3 ± 24.0d	470.7 ± 44.6 ab	551.2 ± 34.5a	301.6 ± 35.3d	425.2 ± 14.2bc	360.9 ± 22.1cd	347.8	448.5	**
cumaric acid	8.51 ± 0.61a	1.22 ± 0.17b	1.05 ± 0.16b	0.63 ± 0.23b	1.31 ± 0.08b	1.19 ± 0.12b	0.89	3.75	**
vanillic acid	87.7 ± 5.6a	47.0 ± 5.6b	53.8 ± 3.6b	46.4 ± 6.2b	49.5 ± 2.9b	51.0 ± 3.9b	48.2	63.6	**
apigenin	7.03 ± 0.81a	1.84 ± 0.56c	2.13 ± 0.24c	3.85 ± 0.27b	1.75 ± 0.32c	4.35 ± 0.60b	2.51	4.47	**
luteolin	63.7 ± 5.7a	47.7 ± 6.1bc	59.8 ± 3.9 ab	55.9 ± 5.1abc	46.1 ± 5.0bc	43.7 ± 5.7c	48.4	57.3	*
luteolin-4-gluc.	115.8 ± 22.1 ab	82.0 ± 2.9c	119.8 ± 16.2a	89.4 ± 3.2abc	77.9 ± 4.1c	84.2 ± 5.7bc	85.1	104.7	*
oleuropein	2350.2 ± 280.4a	688.2 ± 28.7c	841.4 ± 26.1bc	707.9 ± 16.1c	763.8 ± 47.9bc	1066.7 ± 77.4b	765.5	1373.9	**
rutin	84.9 ± 5.8	77.9 ± 3.8	76.0 ± 5.1	76.7 ± 3.7	85.3 ± 6.3	78.6 ± 7.1	76.9	82.9	n.s.
verbascoside	1986.0 ± 101.7c	2327.5 ± 182.7bc	2859.1 ± 193.5a	2493.9 ± 121.5 ab	2538.4 ± 157.9 ab	1985.3 ± 191.5c	2192.1	2537.9	**
ligstroside	428.9 ± 25.4a	265.4 ± 44.9cd	359.2 ± 38.3 ab	240.7 ± 25.1cd	304.7 ± 20.9bc	205.8 ± 29.3d	260.2	341.4	**
oleuropein der.	2792.1 ± 102.2a	1832.1 ± 87.6b	1792.5 ± 127.8b	1272.2 ± 98.9c	1726.0 ± 156.2b	1058.1 ± 68.6c	1461.3	2029.7	**
ligstroside der.	2320.6 ± 148.8a	1180.4 ± 61.1bc	1447.4 ± 131.0b	706.2 ± 22.3d	924.3 ± 29.7cd	1118.0 ± 114.2c	1015.2	1550.5	**
Total phenols	10603.2 ± 580.5a	7181.5± 308.8bc	8337.0 ± 369.2b	6139.9 ± 288.9c	7116.4 ± 406.6c	6196.3 ± 448.0c	6792.2	8399.3	**
Soluble sugars	11.25 ± 0.11a	5.84 ± 0.77bc	4.93 ± 0.54cd	6.04 ± 0.63bc	3.77 ± 0.29d	6.88 ± 0.34b	5.23	7.67	**

Data are expressed as means ± standard deviations. Different letters indicate statistical differences within the same row for Tukey's test. CI= Confidence intervals. **Significance at P ≤ 0.01; *significance at P ≤ 0.05; n.s. not significant.

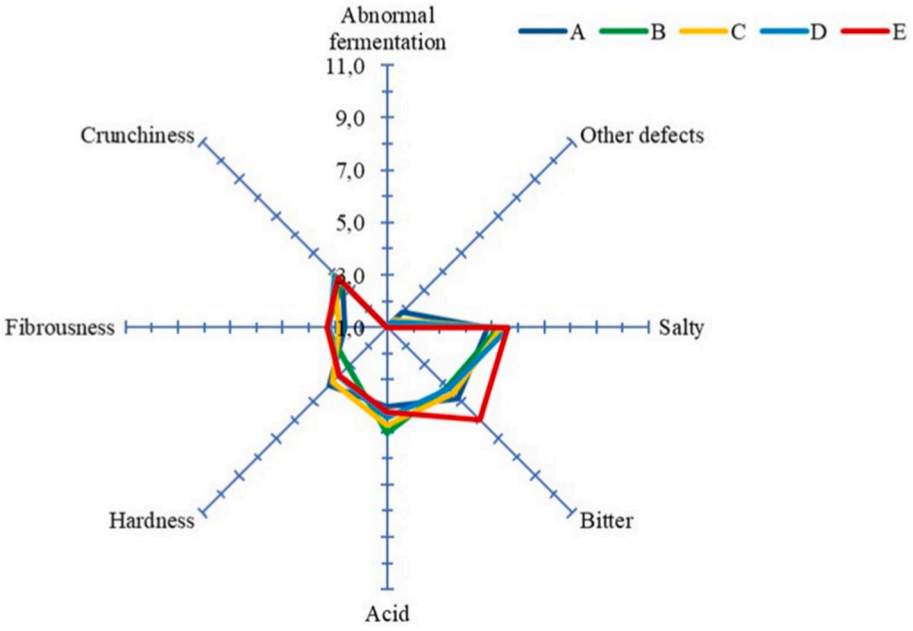

Fig. 3. Evaluation profile of sensory attributes of olive samples with different inocula.

study were obtained at a lab-scale, further studies aimed at scaling up the process will evaluate the industrial application of the selected starter culture. Overall, this study highlighted the relevance to select LAB strains for developing effective starter cultures for producing fermented table olives having appreciated sensorial attributes.

CRediT authorship contribution statement

Paola Foti: Methodology, Conceptualization. **Lara Signorello:** Methodology, Investigation. **Maria Gullo:** Writing – review & editing, Resources, Methodology, Investigation. **Mattia P. Arena:** Formal analysis, Data curation. **Nicolina Timpanaro:** Writing – original draft, Software, Formal analysis. **Cinzia Benincasa:** Writing – original draft, Methodology, Formal analysis. **Innocenzo Muzzalupo:** Writing – review & editing, Supervision. **Flora V. Romeo:** Writing – review & editing, Project administration, Methodology, Funding acquisition.

Statements

All authors agree with the contents of the manuscript and its submission to the journal.

Ethical statements

Participants involved in the sensory analysis were informed about the nature and composition of the experimental product and that the tested product was safe for consumption. Furthermore, appropriate protocols were applied to protect participants' rights and privacy. They gave their consent by signing a document containing information relating to the product and indicating that they could withdraw from the study at any time without giving any reason.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2025.101972.

Data availability

16S rRNA sequence: accession number provided.

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