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# Profiling of phenol content and microbial community dynamics during pâté olive cake fermentation

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ABSTRACT

In this study, different microbial strains, as single and mixed-cultures, were used to ferment the pâté olive cake (POC), a by-product of olive oil processing. In particular, strains belonging to *Candida boidinii, Wickerhamomyces anomalus* and *Lactiplantibacillus plantarum* were used. The fermentation was carried out on diluted (3:2) POC without and with glucose (2% w/v) addition. Furthermore, phenolic compounds were monitored during fermentation in POC added with glucose differetly inoculated and the microbial community, at the end of fermentation, was evaluated by Next Generation Sequencing (NGS) techniques. Data highlighted that inoculated samples showed an hydroxytyrosol content higher than the un-inoculated controls. In particular, during fermentation the sample inoculated with *C. boidinii,* both in single and in mixed culture together with *L. plantarum,* increased the hydroxytyrosol content by 275 and 261 mg/L, respectively, after 8 days, to reach the highest content at the end of fermentation. Metagenomic analysis revealed a low abundance of 16S ribosomal RNA genes and fungal ITS in all samples at any sampling times. Furthermore, at the end of fermentation, all samples exhibited a different bacterial community with a decrease in acetic acid bacteria and an increase in Lactobacillaceae biodiversity. Finally, no effect was detected in any samples on fungal metagenomic profile, where *Dipodascus geotrichum* was found dominant both at initial and final fermentation. In conclusion, the present study confirmed that selected cultures can drive the fermentation and have an impact on the phenolic profile.

## 1. Introduction

Vegetable by-products represent a valuable source for obtaining new products for human consumption, as they are rich in bioactive compounds with nutraceutical traits. In particular, olive oil by-products are a source rich in bioactive compounds, i.e. phenols such as hydroxytyrosol, tyrosol and oleuropein, with confirmed health beneficial effects, contributing to the protection of blood lipids from oxidative stress (EFSA, 2011).

The olive oil extraction technologies include two main systems: the three-phase system, commonly used in Italy and in Greece, which leads mainly to the production of liquid waste, i.e. olive mill wastewater; and the two-phase system, popular in Spain, which leads to the generation of a wet pomace, also called "alperujo" (Uceda et al., 2006; Veneziani et al., 2017). During the olive oil extraction process, only a small part of phenols remains in the olive oil (from 0.5% to 2.0%), while the main portion is concentrated in the liquid and in the solid by-products. Currently, both by-products are reused in various sectors, including agriculture, bioenergy, food and pharmaceutical industry (Ahmed et al., 2019). Recently the two-phase oil extraction system, based on the decanter multiphase (DMF) system (Leopard, Pieralisi, Italy) has been applied to reduce water consumption. Therefore, a dried pomace, with a moisture content of 45%–55%, and a pâté characterized by a high moisture content, between 75% and 90%, consisting of olive pulp and

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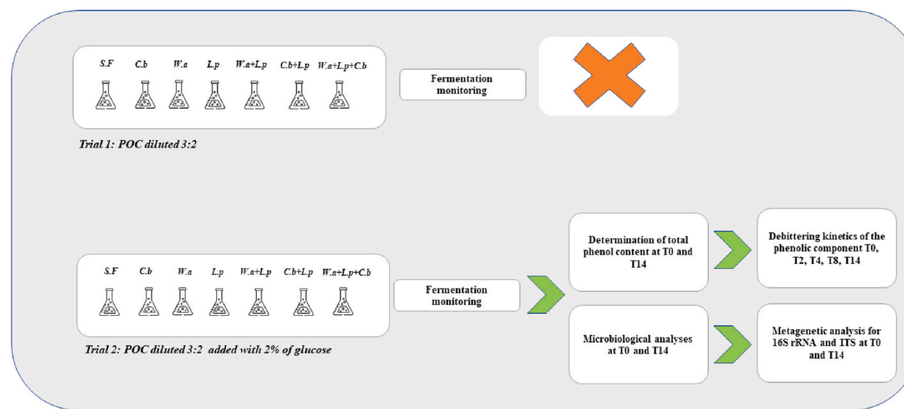


**Fig. 1.** Experimental design.

vegetation water are obtained. While the dried pomace is used as fuel, the pâté, without traces of stones, containing lipophilic and hydrophilic fractions is characterized by interesting technological and functional properties (Foti, Pino, et al., 2022; Lanza et al., 2020; Lozano-Sánchez et al., 2017).

For food application, usually the recovery of phenols is required and their addition in food formulations is applied to provide antioxidant and antimicrobial effects or to extend the shelf life of food products. Otherwise, an alternative approach is to fortify foods by addition of pure extracts, such as hydroxytyrosol and oleuropein, however, this approach is expensive and does not completely solve the problem of by-product management (Caporaso, Formisano, & Genovese, 2018; Foti, Pino, et al., 2022).

The pâté is mainly composed of triterpene acids, hydroxytyrosol, tyrosol, secoiridoid derivatives, verbascoside and fatty acids, such as palmitic acid, oleic acid and polyunsaturated fatty acids. Due to the presence of these compounds, several studies have been focused on the beneficial effects of phenols present in pâté, attributing cardio protective, antiaging and antioxidant activity (Cecchi et al., 2018; Dinu et al., 2020; Peršurić et al., 2020), with an effect on human gastro-intestinal tract (Giuliani et al., 2019). Indeed, spontaneous or driven fermentation is a chance to stabilise and debitter the pâté, improving its nutritional and sensory properties (Durante et al., 2019). In addition, the fermentation driven by selected strains can inhibit the growth of undesirable microorganisms or pathogens reducing spoilage and shortening the debittering process (Perpetuini et al., 2020).

The metagenomic approach is useful for investigating the biodiversity of a microbial community present in a matrix, including food. The NGS techniques of microbial communities has led to the creation of a new scientific field – metagenomics – the analysis of the combined genomes of organisms co-existing in a community. The technique can reveal the main and the secondary microbial groups in fermented foods, providing information on the biodiversity of microbial population on fermentation process and on microbiota dynamics. Furthermore, the advantage of the technique is that it can obviate dependent culture techniques, which are sometimes limiting mainly in complex matrices where microbial cells can be viable but non-culturable (Medina et al., 2016). Recently, to in depth investigate the microbial composition and the metabolic processes that drive the table olives fermentation, omics approaches have been applied (Medina et al., 2016; Vaccalluzzo, Pino, Russo, et al., 2020).

The aim of the present study was to combine the phenol profile detection and the metagenetic approach to insight understand the dynamic of debittering process and the shift of microbial community during fermentation in pâté olive cake differently treated and inoculated with selected lactic acid bacteria and yeast, in single and in mixed cultures.

## 2. Materials and methods

### 2.1. Pâté olive cake and microbial strains

Pâté olive cake (POC), obtained by a multiphase decanter (Leopard, Pieralisi, Jesi, Italy) olive oil extraction, was kindly provided by *Frantoio Oleario Consoli di Consoli Pasquale e Fratelli s.n.c*, an olive oil production company located in Catania, Italy. The samples, obtained in November 2020, from a mix of Nocellara Etnea and Nocellara Messinese cultivars, were stored at −20 °C until use. In the present study, *Candida boidinii* F3 30.1, *Wickerhamomyces anomalus* F5 60.5 and *Lactiplantibacillus plantarum* F 3.5 (DSM 34190, Leibniz-Institute DSMZ, Braunschweig, Germany) strains, isolated from naturally fermented table olives (Pino et al., 2019), belonging to the microbial culture collection of Di3A and ProBioEtna srl, Spin off of University of Catania, were used.

### 2.2. POC antimicrobial activity against yeasts and lactic acid bacteria

The inhibitory activity of POC against yeast and lactic acid bacteria (LAB) strains was evaluated through the disk diffusion method. In detail, the tested microbial cultures were overnight grown on specific media, as Man, Rogosa and Sharpe (MRS, Oxoid, Milan, Italy) for *L. plantarum*, and onto Potato Dextrose Broth (PDB, Merck, Milan, Italy) for yeast strains. Then, using a Mc Farland 0.5, corresponding to a cell density of $10^8$ colony forming unit/mL (CFU/mL) for bacteria and $10^6$ CFU/mL for yeasts, cultures were standardised and 1 mL of each suspension was streaked onto MRSA for *L. plantarum,* and onto Potato Dextrose Agar (PDA, Oxoid) for yeast. Once plates were dried, sterile cellulose discs (Ø 6 mm), imbibed with POC, at different rate dilutions (1:1 to 1:8), were placed on the plates. Distilled water was used as negative control. Plates were incubated at specified temperatures for 48 h and the results were expressed as the diameter of the inhibition halo (mm).

### 2.3. Fermentation trials

In order to evaluate the ability of POC to support the survival/growth of selected strains, fermentation trials were set up. Based on preliminary results (data not shown), untreated POC diluted (3:2) with sterile distilled water, was used. The fermentations were carried out into 250 mL Erlenmeyer flasks, on diluted POC and diluted POC with the addition of 2% (w/v) of glucose (Biolife, Milan, Italy) filtered at 0.22 μm (PTFE filters, Merk, Darmstadt, Germany). Diluted POC was inoculated with a 0.5 Mc Farland standardised microbial cultures (final inoculum volume of 1%) as following: three flasks were inoculated with single cultures (*L. plantarum, W. anomalus, C. boidinii,* at 1% each); two flasks were inoculated with a combined cultures of two strains (*C. boidinii* and *L. plantarum*; *W. anomalus* and *L. plantarum,* at 0.5% each) and one flak was inoculated with a mixed culture of the three strains (*W. anomalus, L.*

*plantarum* and *C. boidinii,* at 0.33% each). Un-inoculated POC was used as control and indicated as spontaneously fermented sample (SF) (Fig. 1). All tests were performed in duplicate. Fermentation was carried out at room temperature (20 ± 1 °C), at a 200 rpm orbital shaking on a rotary plate (Unimax 2010; Heidolph Instruments, Schwabach, Germany). POC samples differently treated were sampled at different times for further analyses. In details, the T0 time was intended as the sampling carried out few hours after POC inoculum with strains.

### 2.4. Chemical analyses

During fermentation, pH was monitored using a Mettler DL25 pH meter (Mettler Toledo International Inc., Columbus, OH, USA) at T0, T8 and T14. The total phenol content in POC samples, at initial (T0) and final fermentation (T14), was determined according to the Folin–Ciocalteu's (FC) colorimetric method. The fermented POC samples, after filtering through Miracloth (Merck), were mixed with 5 mL of FC commercial reagent (Labochimica, Padova, Italy), diluted with water 1:10 (v/v) and blended with 7.5% sodium carbonate solution. After 2 h at room temperature (20 ± 1 °C) in the dark, the solution absorbance was measured spectrophotometrically at 765 nm (Cary 100 Scan UV–Visible, Agilent, Santa Clara, CA, USA). The total phenol content was expressed as mg of gallic acid equivalents (GAE)/L of sample (Romeo et al., 2021; Squillaci et al., 2022). All tests were performed in duplicate.

### 2.5. Microbiological analyses

POC samples were analyzed by serial dilution method in 0.9% (w/v) of sodium chloride sterile solution (Sigma Aldrich, Darmstadt, Germany), by using different selective agar media, and plates were incubated at different conditions. In details, LAB counts were detected into MRSA added with cycloheximide (at a final concentration of 2 mg/mL, Oxoid), and plates incubated at 32 °C for 48 h, under anaerobic conditions; mesophilic aerobic bacteria were detected into Plate Count Agar (PCA, Oxoid), incubated at 32 °C for 48 h; yeasts and moulds were counted into Sabouraud Agar added with chloramphenicol (Bio-Rad, Milan, Italy), incubated at 25 °C for 48 h; staphylococci into Mannitol Salt Agar (MSA, Oxoid) incubated at 32 °C for 72 h; acetic acid bacteria were counted in GYCA (10.0% glucose, 1.0% yeast extract, 1.5% agar) (Oxoid) with 2.0% calcium carbonate, incubated at 30 °C for 3–4 days; coliform bacteria on Chromogenic Coliform Agar Base (Bibby Scharlau, Milan, Italy) incubated at 37 °C for 24 h; Enterobacteriaceae on Violet Red Bile Glucose Agar, aerobically incubated at 37 °C for 24 h, and *Clostridium perfringens* on Sulphite-Polymyxin-Sulphadiazine Agar (SPSA, Oxoid) incubated at 35 °C−37 °C for 18–48 h under anaerobic conditions, by using an Anaerogen kit (Oxoid). The analyses were performed in duplicate and results expressed as Log CFU/g.

### 2.6. Phenolic component in POC samples during fermentation

POC samples were collected at different fermentation times, at initial time (T0) after 2 (T2), 4 (T4), 8 (T8) and 14 (T14) days, in order to evaluate phenolic fraction dynamic through HPLC. The samples were directly filtered into vials with a 0.45 μm filter (PTFE filters, Merk). The instrument used was a Waters Alliance 2695 HPLC liquid chromatography system with a Waters 996 photodiode array (PDA) detector set at 280 nm associated with Waters Empower software (Waters Corporation, Milford, MA, USA). The column maintained at a temperature of 40 °C was a Luna C18 (250 mm × 4.6 mm i.d., 5 μm, 100 Å; Phenomenex, Torrance, CA, USA). Chromatographic separation was achieved by gradient elution using an initial composition of 90% solution A (water acidified with 2% acetic acid) and 10% solution B (methanol) (Merck). Solution B was increased to 30% in 15 min and 70% in 25 min and then, after 2 min in isocratic condition, the mobile phase was set to the initial conditions for 8 min at a flow rate of 1 mL/min. A 50 mM pure gallic acid (Fluka, Losanna, Switzerland) solution was used as internal standard (IS). Identification of phenolic compounds was obtained by comparing the retention time with standard compounds, as: hydroxytyrosol (HT), tyrosol (TYR), chlorogenic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, verbascoside, luteolin-7-o-glucoside, o-coumaric acid, rutin, oleuropein, apigenin-7-o-glucoside, luteolin-4-glucoside, quercetin, luteolin, apigenin (Extrasynthése, Genay, France). All analyses were performed in triplicate.

### 2.7. DNA extraction and high-throughput sequencing by Illumina MiSeq

Total DNA was extracted from different POC samples by using FastDNA spin kit (MP Biomedicals, Solon, OH, USA) and the DNA concentration and quality were detected by a Qubit spectrophotometer (Thermo Scientific, Waltham, USA) and by electrophoresis on 1% agarose gel, respectively. The un-inoculated sample was used as control, while inoculated samples were tested at the end of fermentation (T14). DNA samples were sequenced on an Illumina HiSeq 2500 platform. The V3–V4 hypervariable regions of 16S rRNA gene, for bacteria, and the ITS1-ITS2 region of 18S rRNA gene, for fungi, were amplified by a T100 thermal cycler (Bio-Rad, Hercules, CA, USA). In details the V3/V4Fw (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGA-CAGCCTACGGGNGGCWGCAG - 3′) and V3/V4Rv (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGGACTACHVGGGTATCTAATCC – 3′) and ITSFw (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGA-CAGTCCGTAGGTGAACCTGCGG - 3′) and ITSRv (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGTCCTCCGCTTATTGATATGC - 3′), respectively. The NGS metagenomic analysis was performed by the Nucleotide Sequencing Service at University of Salamanca, Spain.

### 2.8. Bioinformatic data processing

The sequencing quality of the individual samples was validated by the FASTQC software, version 0.11.9, (Andrews, 2010). The adapters used in sequencing were removed from the readings using the cutadapt program (Martin, 2011). A reading filter was performed based on its parameters of quality with the DADA2 program (Callahan et al., 2016): for bacteria the left and right chain was cut at 230 and 220 bases respectively; for fungi a minimum length of 50 bases was established. Biodiversity characterization was carried out using the DADA2 package and the SILVA database, version 138 (Callahan et al., 2016; Quast et al., 2012), for bacteria and UNITE, version 10.05.2021, for fungi (Nilsson et al., 2019). Results were represented by bar diagram R package and Krona software (McMurdie & Holmes, 2013; Ondov et al., 2011). In addition, the Chao1 and Simpson indices were used to assess microbial diversity in POC samples.

### 2.9. Statistical analysis

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

## 3. Results and discussion

### 3.1. Antimicrobial activity and fermentation test

The inhibitory activity of POC, at different dilutions, against the used strains (*L. plantarum, W. anomalus* and *C. boidinii*), was preliminary evaluated and any inhibitory activity was observed (data not shown). This result, in agreement with Tufariello et al. (2019), confirms that the selected strains, isolated from table olive brines, a matrix rich in interfering factors (as polyphenols, and salt), were able to survive and grow

**Table 1**
pH values detected in trial 1 and trial 2 at different fermentation days.

| Trial 1 | Time of fermentation | | |
|---|---|---|---|
| | T0 | T8 | T14 |
| *S. F* | 5.21 ± 0.01 | 5.00 ± 0.01[a] | 4.98 ± 0.01 |
| *C.b* | 5.25 ± 0.07 | 4.94 ± 0.06[abc] | 4.84 ± 0.08 |
| *W.a* | 5.28 ± 0.01 | 4.80 ± 0.02[c] | 4.78 ± 0.01 |
| *L.p* | 5.26 ± 0.01 | 5.00 ± 0.01[a] | 4.88 ± 0.02 |
| *W.a + L.p* | 5.38 ± 0.11 | 4.98 ± 0.01[ab] | 4.97 ± 0.02 |
| *C.b + L.p* | 5.27 ± 0.10 | 4.97 ± 0.02[ab] | 4.96 ± 0.01 |
| *W.a + L.p + C.b* | 5.33 ± 0.05 | 4.84 ± 0.08[bc] | 4.83 ± 0.11 |
| | n.s. | * | n.s. |
| Trial 2 | | | |
| *S. F* | 5.18 ± 0.04 | 4.95 ± 0.07[a] | 4.79 ± 0.01[a] |
| *C.b* | 5.26 ± 0.06 | 4.81 ± 0.01[a] | 4.33 ± 0.01[c] |
| *W.a* | 5.21 ± 0.16 | 4.77 ± 0.03[b] | 4.36 ± 0.01[c] |
| *L.p* | 5.11 ± 0.13 | 4.97 ± 0.02[a] | 4.60 ± 0.01[b] |
| *W.a + L.p* | 5.18 ± 0.11 | 4.87 ± 0.03[ab] | 4.60 ± 0.07[b] |
| *C.b + L.p* | 5.20 ± 0.10 | 4.77 ± 0.01[b] | 4.41 ± 0.03[c] |
| *W.a + L.p + C.b* | 5.25 ± 0.07 | 4.84 ± 0.04[ab] | 4.64 ± 0.05[b] |
| | n.s. | * | ** |

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. n.s. not significant, *Significance at P ≤ 0.05; **Significance at P ≤ 0.01.

in POC without any inhibiting effect. A potential explanation for the ability of *L. plantarum* to grow in presence of HT was suggested by Reverón et al. (2020), who, through a transcriptomic study, demonstrated that *L. plantarum* changes its metabolism to better adapt to the related stressed conditions. Indeed, according to the authors, the microorganism induces antioxidant mechanisms to counteract oxidative damage (including the induction of genes known to be part of the ROS resistome) by expressing genes involved in the response to oxidative stress and genes encoding for $H_2S$ generating enzymes by decreasing the load of copper, a metal that promotes oxidative damage. According to previous reports, *W. anomalus* and *C. boidinii* were frequently detected in olive brines, where they constitute a stable dual species consortium (Pino et al., 2019). In the present study, the fermentation trials were monitored at fixed intervals (T0, T8 and T14) by measuring the pH. As shown in Table 1, different trend in pH values was detected in the two fermentation trials.

In details, the pH, starting from a mean value of 5.14, reached, in trial 1, the lowest mean value of 4.93, after 8 days (T8) to remain slight constant until the 14th day. The initial pH values, detected in the present study, were similar to those described by Lanza et al. (2020) for POC obtained from Leccino and Carboncella cultivar. Regarding the trial 2, a significant constant decrease of pH values was observed in all samples (Table 1). In particular, the pH dropped to 4.33 and 4.36 in samples inoculated with the monoculture of *C. boidinii* and *W. anomalus,* respectively. In the latest case, the inoculation of yeast combined with glucose addition enables the lowering of pH to a safe value, between 4.3 and 4.5 (Perricone et al., 2010). These results highlighted that in the present study the only addition of glucose was able to support the fermentation process, in contrast to Tufariello et al. (2019) that, to start the fermentation process, added yeast extract, peptone and glucose at 0.5% (w/v) into POC obtained from cultivar Cellino di Nardó and Leccino. Based on these results, only POC samples of trial 2 were used for further analyses.

### 3.2. Chemical and microbiological analyses

Through a circolar economy approach, the possible use of olive mill wastewater as plant pre-treated liquid waste and source of water, has been already tested by a few companies (Foti, Occhipinti, et al., 2022). The diluted POC samples subjected to fermentation through selected strains were characterised at the beginning and at the end of fermentation for total phenol content and for the main microbiol groups. At any

**Table 2**
Total phenol content and microbiological counts in POC samples at initial and at the end of fermentation.

| Sample | Time (days) | Total phenolic content (mg/L) | Lactic Acid Bacteria (Log CFU/g) | Yeasts and moulds (Log CFU/g) | Mesophilic aerobic bacteria (Log CFU/g) |
|---|---|---|---|---|---|
| *S. F* | T0 | 1858.07 ± 2.71[d] | 3.00 ± 0.01[bc] | 3.06 ± 0.08[d] | 3.80 ± 0.02[b] |
| *C.b* | T0 | 2046.53 ± 0.54[b] | 3.88 ± 0.57[ab] | 5.15 ± 0.21[a] | 3.50 ± 0.04[bc] |
| *W.a* | T0 | 1757.30 ± 0.57[e] | 3.43 ± 0.21[ab] | 4.57 ± 0.02[ab] | 5.63 ± 0.04[a] |
| *L.p* | T0 | 2560.76 ± 14.14[a] | 3.57 ± 0.02[ab] | 3.23 ± 0.33[d] | 3.06 ± 0.08[bc] |
| *W.a + L.p* | T0 | 1730.38 ± 31.00[e] | 2.10 ± 0.02[c] | 3.35 ± 0.49[bc] | 3.65 ± 0.52[bc] |
| *C.b + L.p* | T0 | 1941.15 ± 1.63[c] | 4.30 ± 0.42[a] | 4.04 ± 0.06[abc] | 3.26 ± 0.37[bc] |
| *W.a + L.p + C.b* | T0 | 2083.07 ± 0.01[b] | 4.04 ± 0.06[ab] | 3.39 ± 0.55[bc] | 2.04 ± 0.06[c] |
| | | ** | * | * | * |
| *S. F* | T14 | 1402.30 ± 1.08[f] | 3.04 ± 0.03[c] | 2.06 ± 0.08[f] | 4.80 ± 0.10[a] |
| *C.b* | T14 | 1853.84 ± 2.17[c] | 3.04 ± 0.05[c] | 3.13 ± 0.01[e] | 3.13 ± 0.13[b] |
| *W.a* | T14 | 2426.15 ± 52.21[a] | 3.45 ± 0.01[abc] | 3.72 ± 0.04[d] | 3.14 ± 0.07[b] |
| *L.p* | T14 | 1516.53 ± 3.80[e] | 3.12 ± 0.18[bc] | 2.24 ± 0.02[f] | 3.10 ± 0.02[b] |
| *W.a + L.p* | T14 | 1651.92 ± 0.54[d] | 3.54 ± 0.02[ab] | 4.79 ± 0.05[a] | 2.50 ± 0.04[c] |
| *C.b + L.p* | T14 | 1805.38 ± 2.17[c] | 3.71 ± 0.02[a] | 4.05 ± 0.08[c] | 2.71 ± 0.01[c] |
| *W.a + L.p + C.b* | T14 | 2007.69 ± 6.52[b] | 3.15 ± 0.21[bc] | 4.41 ± 0.13[b] | 2.41 ± 0.10[c] |
| | | ** | * | ** | ** |

Data are expressed as means ± SD for total phenolic content and as Log CFU/g ± SD for microbial counts. Mean values with different letters within the same column are statistically different. *Significance at P ≤ 0.05; **Significa\nce at P ≤ 0.01.

sampling points the samples were filtered to remove solid residues, such as stone or oil residues, and results are reported in Table 2. The total phenol content at the beginning of fermentation ranged from 1757.30 to 2560.76 mg/L. At the end of fermentation, the total phenol concentration decreased in all samples, except in sample inoculated with *W. anomalus* in single culture, where even an increase was observed, reaching a concentration of 2426.15 mg/L. This result can probably be associated with the strong β-glucosidasic activity of *W. anomalus,* especially at pH close to 5.5 (Bautista-Gallego et al., 2011; Pino et al., 2019; Restuccia et al., 2011; Romeo et al., 2021). Different researchers showed that *W. anomalus,* together with *Debaryomyces hansenii* and *Pichia membranifaciens,* is mostly related to olive matrices thanks to its ability to tolerate extreme environmental conditions such as oxidative, salt, osmotic stress, as well as pH (Padilla et al., 2018). Whereas *C. boidinii* has been frequently exploited for its strong lipase and esterase activities, that positively affect the table olives sensorial traits (Bautista-Gallego et al., 2011).

Overall, microbiological data, obtained by dependent culture techniques, showed low microbial cell density. In details, *Staphylococcus* spp., acetic acid bacteria, coliforms, *E. coli*, enterococci, or *Clostridium perfringens* were undetectable in all samples at both initial and final times, according to Tufariello et al. (2019). The flasks were maintained under shaking during fermentation to allow the growth of LAB and yeasts and different trends for LAB, yeasts and moulds, and mesophilic aerobic bacteria were observed in the differently treated samples at the end of fermentation (Table 2). Regarding LAB, constant densities were observed in all samples at the end of fermentation, except in the samples

**Table 3**
Main phenolic compounds detected at the beginning of fermentation.

| Sample | Vanillic acid (mg/L) | p-Cumaric acid (mg/L) | Oleuropein (mg/L) | Apigenin (mg/L) |
|---|---|---|---|---|
| SF | 68.54 ± 0.73$^{bc}$ | 90.10 ± 0.96$^{abc}$ | 259.79 ± 2.78$^{c}$ | 90.10 ± 0.96$^{abc}$ |
| *C.b* | 96.17 ± 1.14$^{abc}$ | 119.84 ± 11.06$^{a}$ | 409.07 ± 40.53$^{a}$ | 119.84 ± 11.00$^{a}$ |
| *W.a* | 100.00 ± 7.24$^{ab}$ | 123.25 ± 10.35$^{a}$ | 394.08 ± 11.22$^{ab}$ | 123.25 ± 10.35$^{a}$ |
| *L.p* | 106.73 ± 10.87$^{a}$ | 124.21 ± 10.01$^{a}$ | 370.58 ± 37.46$^{ab}$ | 124.21 ± 10.01$^{a}$ |
| *W.a + L.p* | 43.18 ± 0.01$^{c}$ | 47.25 ± 0.02$^{c}$ | 108.29 ± 0.52$^{d}$ | 47.25 ± 0.01$^{c}$ |
| *C.b + L.p* | 70.64 ± 3.23$^{bcd}$ | 69.90 ± 3.80$^{bc}$ | 202.20 ± 14.73$^{cd}$ | 69.90 ± 3.80$^{bc}$ |
| *W.a + L.p + C.b* | 87.56 ± 15.98$^{abc}$ | 106.71 ± 26.53$^{ab}$ | 302.51 ± 33.43$^{bc}$ | 106.71 ± 26.53$^{ab}$ |
| | * | * | ** | * |

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different *Significance at P ≤ 0.05; **Significance at P ≤ 0.01.

**Table 4**
Hydroxytyrosol (HT) and tyrosol (TYR) content in POC samples during fermentation.

| Sample | Time | | | | |
|---|---|---|---|---|---|
| HT (mg/L) | T0 | T2 | T4 | T8 | T14 |
| *S. F* | 297.79 ± 3.19$^{f}$ | 215.95 ± 0.50$^{d}$ | 198.63 ± 4.13$^{c}$ | 99.54 ± 2.29$^{d}$ | 93.83 ± 1.52$^{d}$ |
| *C.b* | 466.65 ± 0.69$^{b}$ | 267.01 ± 2.66$^{b}$ | 196.05 ± 0.76$^{c}$ | 275.83 ± 6.43$^{a}$ | 199.25 ± 4.55$^{b}$ |
| *W.a* | 415.25 ± 4.25$^{c}$ | 264.42 ± 2.61$^{b}$ | 258.07 ± 0.53$^{b}$ | 213.61 ± 0.53$^{b}$ | 132.86 ± 0.96$^{d}$ |
| *L.p* | 462.38 ± 2.00$^{b}$ | 341.86 ± 0.39$^{a}$ | 296.63 ± 5.31$^{a}$ | 215.08 ± 8.27$^{b}$ | 178.74 ± 3.63$^{c}$ |
| *W.a + L.p* | 351.85 ± 0.01$^{e}$ | 211.81 ± 5.23$^{d}$ | 155.21 ± 2.11$^{d}$ | 112.27 ± 3.97$^{d}$ | 96.44 ± 1.13$^{e}$ |
| *C.b + L.p* | 387.85 ± 0.69$^{d}$ | 153.98 ± 3.5$^{e}$ | 131.19 ± 2.60$^{e}$ | 261.18 ± 6.60$^{a}$ | 229.74 ± 1.06$^{a}$ |
| *W.a + L.p + C.b* | 478.47 ± 0.95$^{a}$ | 241.24 ± 0.95$^{c}$ | 119.03 ± 0.76$^{f}$ | 166.01 ± 1.48$^{c}$ | 123.93 ± 1.72$^{e}$ |
| | ** | ** | ** | ** | ** |
| **TYR (mg/L)** | | | | | |
| *S. F* | 136.38 ± 1.40$^{d}$ | 147.58 ± 3.70$^{bcd}$ | 152.98 ± 1.87$^{b}$ | 103.64 ± 3.74$^{d}$ | 122.06 ± 2.47$^{ab}$ |
| *C.b* | 188.94 ± 13.50$^{ab}$ | 140.37 ± 14.40$^{cd}$ | 129.34 ± 7.70$^{c}$ | 206.04 ± 7.57$^{a}$ | 142.87 ± 0.83$^{a}$ |
| *W.a* | 187.04 ± 6.94$^{abc}$ | 162.79 ± 4.93$^{abc}$ | 210.47 ± 7.05$^{a}$ | 182.02 ± 7.04$^{b}$ | 106.89 ± 7.44$^{bc}$ |
| *L.p* | 203.10 ± 2.85$^{a}$ | 198.16 ± 16.73$^{a}$ | 202.80 ± 0.30$^{a}$ | 159.32 ± 1.70$^{c}$ | 123.26 ± 12.89$^{ab}$ |
| *W.a + L.p* | 162.85 ± 0.01$^{c}$ | 161.58 ± 10.20$^{abc}$ | 154.71 ± 2.43$^{b}$ | 109.3 ± 2.47$^{d}$ | 91.04 ± 1.84$^{c}$ |
| *C.b + L.p* | 171.42 ± 4.38$^{bc}$ | 107.05 ± 11.07$^{d}$ | 132.47 ± 0.61$^{c}$ | 157.13 ± 9.64$^{c}$ | 138.51 ± 1.68$^{a}$ |
| *W.a + L.p + C.b* | 206.81 ± 4.67$^{a}$ | 191.90 ± 0.67$^{ab}$ | 118.90 ± 3.47$^{c}$ | 149.13 ± 2.45$^{c}$ | 93.87 ± 8.10$^{c}$ |
| | ** | * | ** | ** | ** |

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. *Significance at P ≤ 0.05; **Significance at P ≤ 0.01.

inoculated with *W. anomalus* and *L. plantarum* in mixed culture, where 1.0 Log unit increase was observed. In details, although the *L. plantarum* F 3.5 (DSM 34190) was inoculated both in single and in mixed cultures in POC samples at an initial density of 8 Log CFU/g any different concentrations were detected in all samples.

Different behavior was observed for yeast counts at both initial and final fermentation, for which higher values were detected in samples inoculated with the two yeast strains, *C. boidinii* F3 30.1 and *W. anomalus* F5 60.5. For these samples 5.15 and 4.57 Log CFU/g initial concentrations were detected, respectively (Table 2). Different values were detected at the end of fermentation, when the higher increases were observed in samples inoculated with *W. anomalus* and *L. plantarum* (1.4 Log unit), followed by samples inoculated with *W. anomalus* and *L. plantarum* and *C. boidinii* (1.0 Log unit). These data confirmed that certain combination of yeasts and LAB promote the lactobacilli growth, as yeasts produce substances, such as vitamins (B1 and B6), amino acids or break down complex carbohydrates that are essential for bacterial growth (Corsetti et al., 2012). In addition, the total mesophilic aerobic bacteria count decreased in all samples during fermentation, except in un-inoculated S.F sample which showed an increase of 1 Log unit at the end of fermentation.

### 3.3. POC phenolic component during fermentation

The dynamic of phenolic profile of POC differently treated at different times during fermentation: T0, T2, T4, T8 and T14 was assessed. The HPLC analysis revealed that at the beginning of the fermentation, different phenols, such as hydroxytyrosol (HT), tyrosol (TYR), oleuropein, vanillic acid, p-coumaric acid and apigenin were found in all samples. In details, while vanillic acid, p-coumaric acid, apigenin and oleuropein were found only at the beginning of fermentation (Table 3), at T2 and T14, the dominance of the two most important phenols of olive by-products, as HT and TYR (Table 4) were found, confirming that the main hydrolysis products of oleuropein include HT and TYR (Charoenprasert & Mitchell, 2012). Moreover, as recently reported, the effect of microbial enzymatic activity in table olives or in olive oil by-products can affect the phenolic profile (Romeo et al., 2021; Vaccalluzzo, Pino, De Angelis, et al., 2020) and, the observed increase of HT content can be related to the used strains. These phenols have been recognised for their antioxidant properties. In detail, the HT (3,4-dihydroxyphenylethanol) is a phenolic alcohol to which multiple activities, including cardioprotective, anticarcinogenic, antimicrobial have been attributed (Parkinson & Cicerale, 2016; Robles-Almazan et al., 2018).

Furthermore, the TYR (2-(4-hydroxyphenyl)-ethanol), a stable compound less prone to autoxidation than other phenols, had shown cellular antioxidant activity and its effect seems to be associated with intracellular accumulation despite its weak antioxidant activity (Di Benedetto et al., 2007; Markovi'c et al., 2019). In the present study, in all inoculated samples, HT concentration decreased at the first two sampling times (T2 and T4), to increase at the last two sampling times (T8 and T14), with the exception of the un-inoculated sample (S.F). A significant increase occurs at the 8th day in sample inoculated with *C. boidinii* in single culture and in association with *L. plantarum*, reaching, after 8 days, concentrations of 275 mg/L and 261 mg/L, respectively. The increase of the HT concentration during fermentation driven by yeasts could be explained, according to Fernández de Ullivarri, Mendoza, and Raya (2018), which demonstrated that in wine yeasts can produce higher concentrations of HT, as a secondary metabolite from tyrosine, and not only from the degradation of other polyphenolic compounds, such as anthocyanins or oleuropein (Charoenprasert & Mitchell, 2012; Motilva et al., 2016).

These data confirm that selected yeasts can promote LAB growth as well as the phenolic compounds degradation thanks to their β-glucosidasic activity (Corsetti et al., 2012). It is interesting to note that the association of the two yeasts together with *L. plantarum* slightly increased the concentration of HT. This phenomenon is probably due to competition for the carbon source between the two yeasts during fermentation. Indeed, authors claim that *W. anomalus* possesses a 'killer activity' against several yeasts, exhibiting a biocontrol action against both spoilage non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae* strains (Csutak et al., 2017; Fernández de Ullivarri, Mendoza, & Raya,

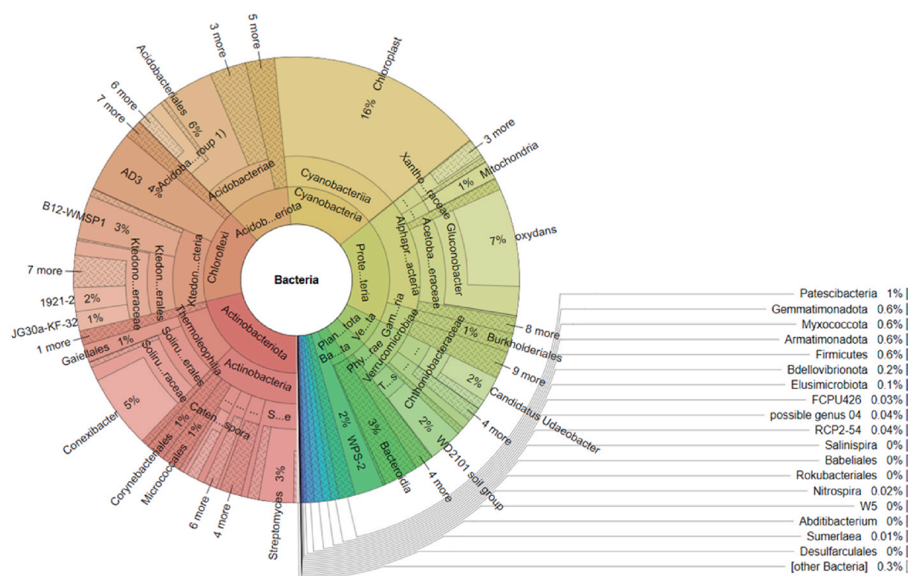


**Fig. 2.** Krona graphical representation of taxonomic abundance in S.F sample at initial time.

2018; Liu et., 2015).

Regarding TYR, the highest concentrations were reached in the *C. boidinii* single-culture samples, after 8 days, and in *W. anomalus* and *L. plantarum,* at T4, reaching concentrations of 206.04 mg/L, 210.47 mg/L and 202.80 mg/L, respectively. However, a similar TYR concentration (206.81 mg/L) was reached at the first sampling time in sample co-inoculated with the three cultures (Table 4).

Recently, to calculate olive oil polyphenol for applying the EFSA health claim, some authors have stressed the need to quantify aside from the HT, also the total TYR content (Bellumori et al., 2019). In the case of TYR, a higher concentration in the samples could also be related to the presence of yeasts. In fact, several studies use yeasts, such as *S. cerevisiae,* or modify the biosynthesis of TYR (via shikimate and the L-tyrosine branch) to increase the production of this compound (Guo et al., 2020; Liu et al., 2021).

### 3.4. Diversity and change of microbial community during fermentation

POC samples, both controls and inoculated, were subjected to sequencing of the ITS region and 16S rRNA. The control sample (S.F) was sequenced at initial time (T0) in order to mark the shift in microbial population at the end of fermentation.

The Chao1 index showed, for the ITS profile, a total number of 61 determined genera, with an average number of 7.625 genera per sample. In particular, for the ITS region, both Chao1 and Simpson indices highlighted that the *C. boidinii* + *L. plantarum* sample showed the highest number of genera (13 and 0.82, respectively) at the end of fermentation (Fig. S1).

As far as 16S rRNA gene is concerned, on the other hand, several species, in a range between 500 and 1050, were detected. Using the Chao1 and Simpson indices, the total number of found species was 5819 with an average per sample of 727.375 species (data not shown), and highest number of species was found in samples inoculated with *W. anomalus* + *L. plantarum* + *C. boidinii* (Fig. S2).

Concerning the ITS biota, the results showed the dominance of *Dipodascus geotrichum,* a common yeast-like fungus, found in all samples both before and after the fermentation process (data not shown). *D. geotrichum* is a ubiquitous fungus commonly isolated form different habitats rich in sugars or other nutrients. The species has been isolated from soil, air, water, milk, silage, plant tissue and the digestive tract of humans and other mammals. Thermophilic species may be found colonizing the intestinal and respiratory tracts of mammals and may provoke disorders in immunocompromised patient (Pottier et al., 2008). In the food industry, *D. geotrichum* is a dominant yeast in dairy products and appears to exert a functional action related to sugars, milk fat and proteins (Kačániová et al., 2021) and flavour production (Grondin et., 2017). Only in the samples inoculated with *C. boidinii* + *L. plantarum* and in samples inoculated with the mix of the three strains, a slight change in fungal population occurred at the end of fermentation. Indeed, in samples inoculated with *C. boidinii* + *L. plantarum* the presence of Basidiomycota (2%), and specifically Agaricomycetes, was found. These fungi include the industrially cultivated for edible and medicinal mushrooms (Merényi et al., 2022). While in the sample inoculated with the three-strain combo, 0.6% of Metschnikowiaceae was found. This microbial group is known to be present in fermented foods and species belonging to *Metschnikowia* genus have shown to actively affect the aromatic profile of wines, as they induce a higher production of ethyl esters and long-chain fatty acids (Blanco et al., 2021; Wang et al., 2022). The metagenomic analysis performed highlighted in which way the inocula influenced also the indigenous microbial community of the POC analyzed. These results showed that species, as *D. geotrichum,* already present in the initial matrix remain dominant until the end of fermentation.

The bacterial microbioma showed a higher variable profile, compared to fungi (Fig. S3), although the highest relative abundance (at 0.3) occurred in the sample at the beginning of fermentation (S.F at T0). The NSG analyses also revealed between 32% and 42% of unidentified genera.

The detected phyla in POC samples, showed with KRONA software, before fermentation, are shown in Fig. 2. In details, Actinobacteriota (21%), Proteobacteria (18%), Chloroflexi (16%), Acidobacteriota (11%), Cyanobacteria (16%) were detected. These phyla have been found as naturally present both in soil and in aquatic ecosystems (Mutoti et al., 2022; Wang et al., 2019).

Interestingly, before the addition of selected microbial cultures, within the Acetobacteriaceae family, *Gluconobacter oxydans* was found as a dominant species (7%). Different strains of the species have been used for long time in biotechnology industry for their ability to oxidise various compounds such as sugars, polyols and alcohols, as well as ketones and aldehydes and for the production of bioactive compounds, including L-ascorbic acid (vitamin C) (Da Silva et al., 2022).

At the end of fermentation (at T14), in all samples, including control sample (S.F), a decrease in Acetobacteriaceae occurred, with the highest decrease detected for *G. oxydans,* that reached 0.02% in sample

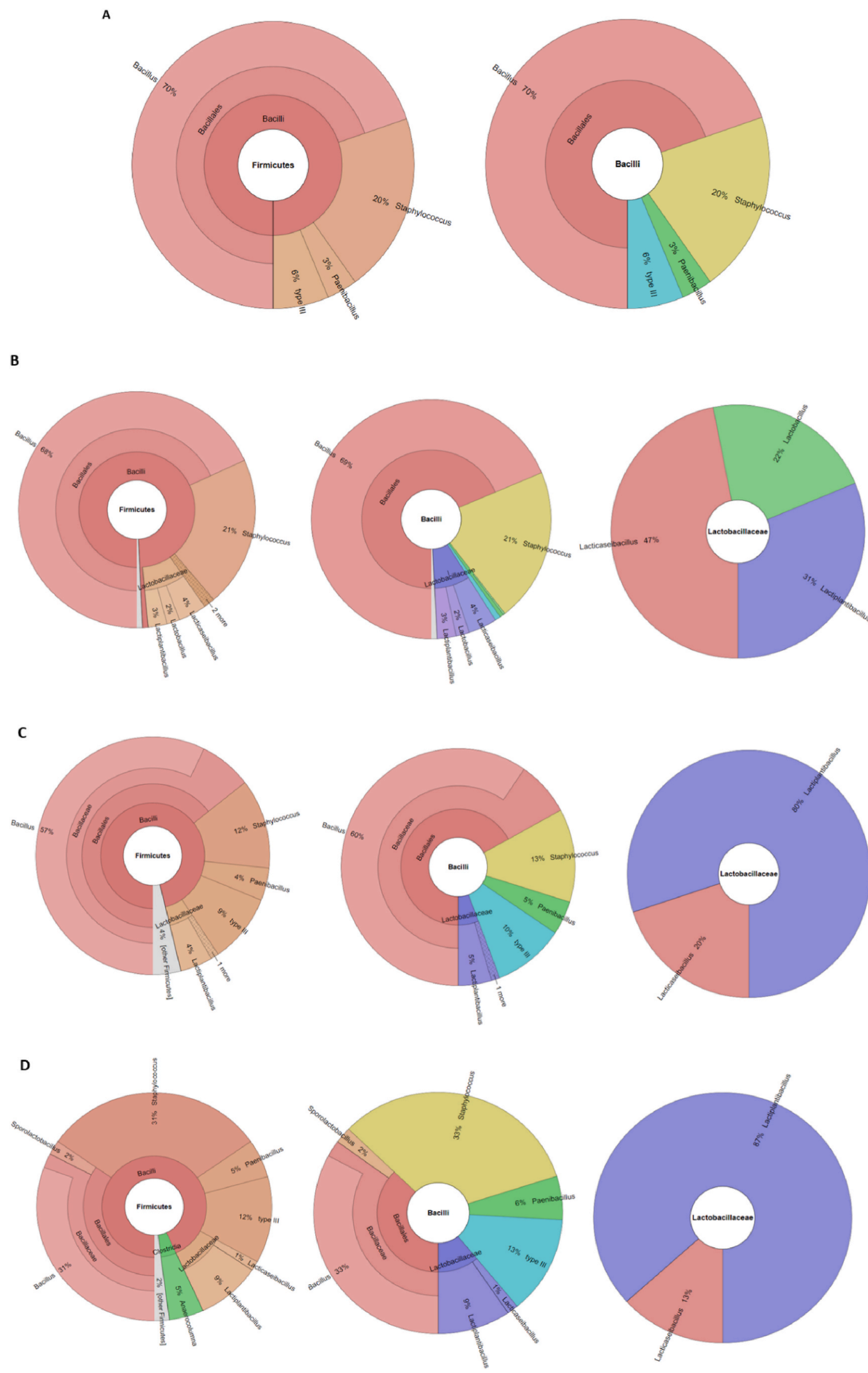


**Fig. 3.** Krona graphical representation of taxonomic Firmicutes abundance **A:** S.F T0 (Spontaneus fermentation); **B**: S.F T14 (Spontaneus fermentation); **C**: sample inoculated with *L. plantarum* T14; **D:** sample inoculated with *W. anomanlus* and *L. plantarum.*

inoculated with *C. boidinii* + *L. plantarum*. Although difficult to cultivate, isolate and identify, acetic acid bacteria represent promising starter cultures to better control food fermentation processes but most importantly to produce new fermented foods and beverages (De Roos & De Vuyst, 2018).

Zooming on Firmicutes phylum profile, only the 0.6% was found in samples at the beginning of fermentation (Fig. 2) while no Lactobacillaceae was revealed (Fig. 3A). This percentage remained almost unchanged up to the end of fermentation, although a slight different composition in Firmicutes was detected at the end of fermentation. Indeed, at the end of fermentation, the S.F sample showed the presence of 3% of *Lactiplantibacillus* spp., 4% of *Lacticaseibacillus* and 2% of *Lactobacillus jensenii* (Fig. 3B). *Lactiplantibacillus* was mainly found (5%) in samples inoculated with *L. plantarum* (Fig. 3C) and in the samples inoculated with the combination of *W. anomalus* and *L. plantarum* (9%) (Fig. 3D), indicating the survival of *L. plantarum* selected strain in this matrix. Zooming on the Lactobacillaceae composition, in latter two samples, an increase on *Lactiplantibacillus* community was riveled, up to 80% and 87%, respectively (Fig. 3C and D).

Although the low abbundance of LAB in plant microbiota, as in the case of POC, there is a growing interest in the scientific community in use LAB in plant by-products to obtain functional products, rich in bioactive compounds, thanks the ability of LAB to grow and adapt to different plant niches (Sabater, Ruiz, Delgado, Ruas-Madiedo, & Margolles, 2020).

## 4. Conclusion

The aim of this study was to monitor the fermentation process of POC inoculated with microbial strains in different combinations in order to propose a strategy to improve the healthy value of such a by-product. Results on phenolic component showed that the use of microbial monocultures or mixed cultures can influence the profile of bioactive compounds during fermentation. Moreover, NGS analyses applied to in depht explore and better understand the function of microbial community, revealed a shift on bacterial population whereas lower effect were detected for yeast population. Moreover, results of the present study confirmed that POC can be considered microbiologically safe and a relevant source to be used as food and/or ingredient. Further studies will be conducted on a larger volume in order to standardise and replicate an industrial scale-up process to obtain new environmentally friendly foods or functional ingredients.

## CRediT authorship contribution statement

Paola Foti, Nunziatina Russo, Flora V. Romeo: Investigation, Methodology, Writing – original draft; Paola Foti, Flora V. Romeo, Alessandra Pino, Altino Branco Choupina: Software, Methodology; Cinzia Caggia, Flora V. Romeo: Conceptualization, review & editing; Cinzia L. Randazzo: Conceptualization, Writing – review & editing; Cinzia Caggia: Conceptualization, Supervision, Funding acquisition, Review & editing. All authors have read and agreed to the published version of the manuscript.

## 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

No data was used for the research described in the article.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102358.

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