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# Quantitative PCR Assay as a Tool for the Detection of Lactobacilli in Sicilian Table Olives Produced at an Industrial Scale

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**Abstract:** Table olives are an important fermented product of the Mediterranean area consumed all over the world. In our era, the food industry requires a safe and stable final product with desirable characteristics for the consumer. In the present study, two different experimental fermentations (L, with *Lactiplantibacillus plantarum* strains, and LY, with *L. plantarum* strains and *Wickerhamomyces anomalus* strain) were conducted and monitored up to 180 days and compared with a spontaneous fermentation, used as control (C). The safety and stability of table olives were determined by applying a plate count and quantitative real-time PCR (qPCR) approach. Compared with the control sample (C), experimental fermentations showed a faster acidification and a good inhibition rate of spoilage bacteria, indicating the safety of the process. Quantitative PCR data confirmed the abundance of the *Lactobacillus* group in both experimental table olives, confirming the importance of the starter cultures for the stability of the final product. In conclusion, the use of starter cultures ensures the safety of industrially produced table olives, and the application of qPCR seems to be a promising tool to detect and quantify lactobacilli as a positive biomarker of table olive fermentation.

**Keywords:** table olives; starter cultures; *L. plantarum*; *W. anomalus*; quantitative real-time PCR; lactic acid bacteria; yeast



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## 1. Introduction

According to the International Olive Oil Council (IOOC), table olives are defined as "the product obtained from the healthy fruit of cultivated olive varieties (*Olea europaea* L.) selected for their olive production whose volume, shape, flesh-to-stone ratio, fine flesh, flavour, taste, firmness and ease of detachment from the stone make them particularly suitable for processing" [1]. Table olives are fermented foods, resulting from the microbial bioconversion of a raw material into an edible product suitable for human consumption, which have a great global socio-economic impact, especially in Mediterranean countries (e.g., Spain, Greece, Italy, and Portugal) [2]. Several processing methods, such as Sevillian, Greek, and Californian styles, with or without lye treatment, are currently applied to produce table olives. Specifically, in Sicily drupes are directly immersed into brine and the fermentation process is carried out by the autochthonous microbiota without the use of starters and debittering pre-treatments [3]. Several parameters, such as temperature and salt concentration of the brine. strongly affect the fermentation process of table olives and, in turn, the composition of the final product [4–6]. In addition, physico-chemical conditions and availability of fermentable substrates contribute to reduce the reproducibility of the process, leading to final products with different properties, quality levels, and consumer

acceptance. For this reason, the use of starter cultures is strongly recommended, especially at the industrial level, allowing a greater control of the fermentation and a high stability of the final product [7]. Starter cultures could accelerate the hydrolysis of bitter compounds, improve the aroma of the final product, influencing taste, texture, and safety of the final product [8–10]. Autochthonous strains, which naturally dominate the spontaneous fermentation, tend to have high metabolic capacities, beneficially affecting the quality of the final product. Nowadays, several studies have been conducted in order to select multifunctional starters of lactobacilli and yeast strains, able to accelerate the brine acidification, to inhibit pathogenic and/or spoilage microorganisms and to enhance the sensory profile of the final products [11]. According to that, a wide range of microorganisms, including lactobacilli, such as *Lactiplantibacillus plantarum* and *Lactiplantibacillus pentosus* species, as well as yeasts, mainly belonging to *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, and *Candida boidinii* species were widely used as starters. In particular, the use of strains belonging to the *Lpb. plantarum* species, alone or in combination with yeasts, appears to be a highly promising biotechnological tool for table olive fermentation [9]. To date, several studies shed light on the complex microbiota of table olives. Indeed, culture-dependent and independent techniques were extensively applied to detect and understand the role of the microbiota involved in the fermentation process [3,12,13]. Among molecular methods, the quantitative real-time PCR (qPCR) is widely used for the detection and quantification of bacteria associated with complex ecosystems. This technique has a faster detection rate and high sensitivity, compared with conventional PCR. In addition, it is highly specific and reproducible over time and is often applied to shed light on the microbial composition of different matrices allowing the detection of dominant and spoilage bacteria in food [14,15]. It is well known that the qPCR is particularly suitable to study the bacterial communities associated to fermented foods, which are characterized by the presence of a limited number of core species [16]. Nevertheless, based on our knowledge, only one study deals with the application of this technique to the olive microbiota [17].

According to that, the aim of the present work is to reveal the dominance of the lactobacilli population through the fermentation process of Nocellara Etnea table olives, produced at an industrial scale, by using qPCR. In addition, the effect of selected starter cultures on both microbiological and physico-chemical parameters of table olives was investigated.

## 2. Materials and Methods

### *2.1. Experimental Fermentations*

Olives of the Nocellara Etnea cultivar were processed at the local company Giuseppe Rosso Soc. Agr. Unipersonale srl (Ragusa, Italy). Olives were transferred to polyethylene containers (180 L volume) and directly immersed in a marine salt solution (7% NaCl *w*/*v*). Strains ascribed to the *Lpb. Plantarum* and *W. anomalus* species were used as starter cultures for the experimental fermentations. In detail, the *Lpb. Plantarum* F3.5 and C11C8 strains were previously isolated from brine samples and characterized for technological aptitude, ability to degrade oleuropein [10,18,19], and probiotic features. The *W. anomalus* F5.60.5 strain was previously isolated from naturally fermented table olives [19] and tested for tolerance to stressed brine conditions and oleuropein reduction. Two different experimental fermentations, L and LY, were compared. In detail, the L fermentation was prepared by using a blend of the F3.5 and C11C8 strains (ratio 1:1) and inoculated at the final cell density of 7 log CFU/mL, whereas for the set-up of the LY fermentation, the F5.60.5 strain, standardized to a final cell density of 5 log CFU/mL, was combined with the aforementioned blend of lactobacilli. Fermentation batches without starter cultures were used as control samples (C). All fermentations were carried out at room temperature ($18 \pm 2$ °C) and monitored for a total of 180 days. In addition, marine salt was periodically added to maintain constant the concentration of sodium chloride. Each fermentation was carried out in triplicate.

### 2.2. Microbiological and Chemical Analyses

Microbiological analyses were performed on both brine and olives samples before the starter culture addition (T0) and after 7, 30, 60, 90, 120, and 180 days of fermentation according to the method proposed by Vaccalluzzo and co-workers [20]. In detail, the drupes were carefully separated from the brine samples, drained, pitted, and 25 g were transferred into a sterile stomacher bag, diluted with Ringer's solution (Sigma-Aldrich, Milan, Italy) and homogenized for 2–5 min in a stomacher (Lab-Blender, Seward, London). Both brine (10 mL) and olive samples, homogenized as reported above, were serially diluted, using sterile quarter-strength Ringer's solution (QRS), and plated on the following agar media and conditions: Plate Count Agar, incubated at 32 ± 2 °C for 48 h, for total mesophilic bacteria; de Man-Rogosa-Sharp agar, supplemented with cycloheximide (5 mL/L), anaerobically incubated at 32 °C for 24–48 h, for LAB count; Sabouraud Dextrose Agar, supplemented with chloramphenicol (0.05 g/L), incubated at 25 °C for 4 days, for yeast count; Violet Red Bile Glucose Agar, aerobically incubated at 30 °C for 18–24 h, for Enterobacteriaceae count; Mannitol Salt Agar, incubated at 32 °C for 48 h, for staphylococci enumeration; Mac Conkey incubated at 32 °C for 24–48 h for Escherichia coli determination; Sulphite Polymyxin Suphadiazine Agar, anaerobically incubated at 37 °C for 24–48 h, for the detection of sulfite-reducing clostridia, as *Clostridium perfringens* species, and Bacillus cereus Agar (Mossel) was used for the detection of *Bacillus cereus* spp. In addition, a protocol proposed by Randazzo and co-workers [21] was followed for the detection of pathogenic foodborne species such as *Listeria monocytogenes* and *Salmonella* spp. All media were purchased from Liofilchem (Roseto degli Abruzzi, Italy). All analyses were performed in triplicate. Results were expressed as log10 CFU/mL for brine and log10 CFU/g for olive samples and standard deviation.

The pH of the brine samples was measured, during the fermentation process (0, 7, 30, 60, 90, 120, and 180 days), using a MettlerDL25 pHmeter (MettlerDL25, Mettler-Toledo International Inc.). The NaCl salt content was monitored following the method proposed by Benítez-Cabello and co-workers [22].

### 2.3. Total gDNA Extraction and Detection of Lactobacilli by qPCR

Olives samples collected at 7, 90, and 180 days of fermentation from L, LY, and C samples, were subjected to gDNA extraction using the Dneasy Mericon Food Kit (Qiagen, Milan, Italy) and according to the protocol reported by Vaccalluzzo and co-workers [20] and Pino and co-workers [23]. DNA concentration was measured using the fluorimeter Qubit 4.0 (Invitrogen, Carlsbad, CA, United States). In order to quantify the lactobacilli population, the gDNA was subjected to quantitative PCR (qPCR) following the method proposed by Bornes and co-workers [24]. The qPCR reactions were performed using the QuantiFast SYBR Green qPCR kit (Qiagen, Milan, Italy) on a Rotor Gene Q instrument (Qiagen, Milan, Italy). The primer pair, targeting the *Lactobacillus* genus (LacF 5′-CGATGAGTGCTAGGTGTTGGA-3′ and LacR 5′-CAAGATGTCAAGACCTGG TAAG-3′), with an amplicon size of 186 bp, was used. In detail, the qPCR mix was performed in a final volume of 25 µL, containing 12.5 µL of QuantiFast SYBR Green Mastermix, 10 µM of both forward and reverse primers, 1 µL of the gDNA template, and 9.5 µL of water. The cycling conditions included an initial holding phase at 94 °C for 15 min followed by 40 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. To ensure amplification specificity, a melting curve analysis was performed considering a temperature range between 70 °C to 95 °C. To validate the reaction, a standard curve was generated using the genetic material isolated from a pool of lactobacilli in a range from $10^9$ to $10^3$ CFU/mL. All reactions were processed in three different replicates.

### 2.4. Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied to both pH values and microbiological data using the Statistica software (version 10.0 for

Windows, TIBCO Software, Palo Alto, CA, USA). Differences were considered statistically significant at $p < 0.05$.

## 3. Results

### *3.1. pH Values of Experimental Brine Samples*

Table 1 shows the pH values measured during the fermentation process of the experimental table olives, with (L and LY) and without (C) the addition of starter cultures. At T0 sampling time, no significant differences were recorded among samples, with an average pH value of 6.67. Starting from the 7th day of fermentation, a decreasing trend in pH was observed in all experimental samples although a more pronounced decrease was detected in both L and LY samples. In particular, 30 days after the start of the fermentation process, L and LY samples reached a pH of 4.34 and 4.69 respectively, while the C samples exhibited a pH value of 5.18 (Table 1). At the end of the fermentation process (180 days), the inoculated experimental samples L and LY showed pH values of 3.90 and 4.17 respectively, which are below the threshold values of 4.30 recommended by the International Olive Oil Council (IOOC) [1]. Differently, control samples, with a pH of 4.69, did not reach, at 180 days of fermentation, the value recommended by IOOC.

**Table 1.** pH values of brine samples of Nocellara Etnea table olives produced at an industrial scale with (L and LY) and without (C) stater culture addition.

| | pH | | | | | | |
|---|---|---|---|---|---|---|---|
| **Samples** | **T0** | **T7** | **T30** | **T60** | **T90** | **T120** | **T180** |
| **C** | $6.67 \pm 0.10^{aA}$ | $5.80 \pm 0.30^{aB}$ | $5.18 \pm 0.08^{aC}$ | $5.04 \pm 0.14^{aCD}$ | $5.03 \pm 0.05^{aCD}$ | $4.91 \pm 0.05^{aCD}$ | $4.69 \pm 0.03^{aD}$ |
| **L** | $6.64 \pm 0.07^{aA}$ | $5.06 \pm 0.08^{bB}$ | $4.34 \pm 0.03^{cC}$ | $4.17 \pm 0.04^{bCD}$ | $3.93 \pm 0.08^{bD}$ | $3.80 \pm 0.09^{cE}$ | $3.90 \pm 0.13^{bDE}$ |
| **LY** | $6.69 \pm 0.06^{aA}$ | $5.11 \pm 0.06^{bB}$ | $4.69 \pm 0.05^{bC}$ | $4.22 \pm 0.06^{bCD}$ | $4.13 \pm 0.04^{bD}$ | $4.17 \pm 0.09^{bD}$ | $4.17 \pm 0.09^{bD}$ |

Results are reported as average value and standard deviation of three replicates. $^{a–c}$ Different superscript letters within the same column indicate significant differences at $p < 0.05$. $^{A–E}$ Different superscript letters within the same row indicate significant differences at $p < 0.05$.

### *3.2. Microbial Data of Experimental Brine and Olive Samples*

Table 2 reports the microbial count values, expressed as CFU/mL and CFU/g, for the brine and drupe samples, respectively. The experimental samples were monitored at 0, 7, 30, 60, 90, 120, and 180 days. Up to now, no official microbiological criteria for fermented table olives are provided [25]. Nevertheless, based on the minimum hygiene requirements of the Codex Alimentarius standards and the suggestions of European Regulation No. 2073/2005, spoilage species must be less than 100 CFU/g and pathogenic species must be absent in 25 g of product. According to that, in the present study, based on culture dependent data, *Bacillus cereus*, sulfite-reducing clostridia such as *Clostridium perfringens*, and *Escherichia coli*, as well as the foodborne pathogens *Listeria monocytogenes* and *Salmonella* spp., were not detected in the analyzed samples.

**Table 2.** Microbial counts detected on brine and olive samples of Nocellara Etnea cultivar v.

| | Brine | | | | | | | Olive | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **0** | **7** | **30** | **60** | **90** | **120** | **180** | **0** | **7** | **30** | **60** | **90** | **120** | **180** |
| | | | | | | | **Mesophilic Bacteria** | | | | | | | |
| **C** | 5.55 ± 0.56 aB | 6.42 ± 0.08 aA | 6.28 ± 0.04 aA | 6.23 ± 0.03 aA | 6.27 ± 0.08 aA | 5.60 ± 0.04 aB | 4.45 ± 0.05 aC | 4.84 ± 0.04 aE | 5.52 ± 0.06 aD | 7.28 ± 0.04 aB | 7.69 ± 0.12 aA | 6.16 ± 0.05 aC | 5.46 ± 0.04 aD | 5.06 ± 0.04 aE |
| **L** | 5.46 ± 0.14 abB | 6.15 ± 0.07 bA | 5.49 ± 0.06 cB | 4.57 ± 0.05 bC | 3.83 ± 0.13 bD | 3.19 ± 0.03 bE | 3.15 ± 0.04 bE | 4.93 ± 0.04 aD | 5.29 ± 0.13 aC | 5.74 ± 0.06 bB | 6.59 ± 0.05 bA | 4.57 ± 0.03 cE | 3.28 ± 0.08 bF | 3.13 ± 0.04 bF |
| **LY** | 5.86 ± 0.14 aB | 6.20 ± 0.04 abA | 5.89 ± 0.09 bAB | 4.34 ± 0.06 cC | 4.04 ± 0.08 bC | 3.26 ± 0.08 bD | 3.17 ± 0.04 bD | 4.87 ± 0.17 aD | 5.38 ± 0.08 aBC | 5.69 ± 0.07 bB | 6.74 ± 0.06 bA | 5.07 ± 0.05 aCD | 3.54 ± 0.05 bE | 3.24 ± 0.04 bE |
| | | | | | | | **Lactic acid bacteria** | | | | | | | |
| **C** | 3.64 ± 0.06 aC | 3.67 ± 0.04 bC | 4.32 ± 0.11 bB | 5.10 ± 0.03 cA | 5.14 ± 0.05 cA | 4.24 ± 0.05 bB | 4.13 ± 0.04 cB | 3.55 ± 0.16 aE | 4.42 ± 0.17 bCD | 4.89 ± 0.05 cAB | 4.52 ± 0.07 bBC | 5.17 ± 0.09 bA | 4.94 ± 0.06 bAB | 4.00 ± 0.15 bD |
| **L** | 3.66 ± 0.07 aF | 7.84 ± 0.02 aBC | 7.96 ± 0.06 aB | 8.29 ± 0.05 aA | 7.58 ± 0.05 bC | 6.78 ± 0.16 aD | 6.14 ± 0.02 bE | 3.44 ± 0.08 aF | 7.63 ± 0.10 aCD | 7.91 ± 0.06 bBC | 8.25 ± 0.06 aA | 7.97 ± 0.09 aAB | 7.59 ± 0.08 aD | 7.11 ± 0.04 aE |
| **LY** | 3.67 ± 0.07 aE | 7.93 ± 0.06 aAB | 8.04 ± 0.06 aA | 7.87 ± 0.04 bAB | 7.79 ± 0.04 aB | 7.13 ± 0.04 aC | 6.63 ± 0.07 aD | 3.62 ± 0.16 aC | 7.85 ± 0.07 aAB | 8.18 ± 0.05 aA | 8.19 ± 0.07 aA | 8.11 ± 0.01 aA | 7.90 ± 0.16 aAB | 7.35 ± 0.05 aB |
| | | | | | | | **Staphylococci** | | | | | | | |
| **C** | 4.23 ± 0.05 aB | 4.80 ± 0.04 aA | 4.29 ± 0.05 aAB | 3.36 ± 0.20 C | 2.35 ± 0.21 D | <1 | <1 | 4.12 ± 0.04 aB | 4.61 ± 0.04 aA | 4.12 ± 0.16 aB | 3.40 ± 0.18 C | 3.41 ± 0.19 C | 2.02 ± 0.11 D | <1 |
| **L** | 4.27 ± 0.04 aA | 3.63 ± 0.04 bB | 2.67 ± 0.05 bC | <1 | <1 | <1 | <1 | 4.07 ± 0.07 aA | 3.62 ± 0.09 bA | 2.15 ± 0.21 bB | <1 | <1 | <1 | <1 |
| **LY** | 4.26 ± 0.05 aA | 3.72 ± 0.03 bB | 2.57 ± 0.07 bC | <1 | <1 | <1 | <1 | 4.12 ± 0.06 aA | 3.48 ± 0.04 bB | 2.18 ± 0.04 bC | <1 | <1 | <1 | <1 |
| | | | | | | | **Enterobacteriaceae** | | | | | | | |
| **C** | 3.62 ± 0.12 aD | 5.38 ± 0.09 aA | 5.06 ± 0.09 aB | 4.57 ± 0.05 aC | 3.36 ± 0.04 D | 2.77 ± 0.05 E | <1 | 3.25 ± 0.07 aB | 4.43 ± 0.18 aA | 4.30 ± 0.14 aA | 3.31 ± 0.27 aB | 2.37 ± 0.06 C | 2.17 ± 0.09 C | <1 |
| **L** | 3.63 ± 0.10 aB | 4.19 ± 0.05 bA | 3.14 ± 0.06 bC | 2.23 ± 0.11 bD | <1 | <1 | <1 | 3.14 ± 0.05 aB | 4.11 ± 0.09 aA | 3.19 ± 0.10 bB | 2.21 ± 0.13 bC | <1 | <1 | <1 |
| **LY** | 3.62 ± 0.08 aB | 4.17 ± 0.09 bA | 2.27 ± 0.04 cC | 2.08 ± 0.06 bC | <1 | <1 | <1 | 3.12 ± 0.18 aB | 4.18 ± 0.10 aA | 3.24 ± 0.06 bB | 2.24 ± 0.04 bC | <1 | <1 | <1 |
| | | | | | | | **Yeasts** | | | | | | | |
| **C** | 3.99 ± 0.07 aD | 4.54 ± 0.12 bC | 5.74 ± 0.06 bA | 5.21 ± 0.05 bB | 4.51 ± 0.09 bC | 3.64 ± 0.04 bE | 3.37 ± 0.04 bE | 2.72 ± 0.12 aF | 4.85 ± 0.07 bB | 5.28 ± 0.06 bA | 5.21 ± 0.16 bAB | 4.31 ± 0.09 bC | 3.86 ± 0.06 bD | 3.15 ± 0.06 bE |
| **L** | 3.89 ± 0.08 aBCD | 3.61 ± 0.21 cCD | 4.87 ± 0.05 cA | 4.14 ± 0.04 cB | 4.03 ± 0.04 cBC | 3.59 ± 0.12 bD | 3.15 ± 0.07 bE | 2.65 ± 0.19 aD | 4.41 ± 0.05 cB | 5.04 ± 0.04 cA | 5.18 ± 0.08 bA | 4.25 ± 0.04 bB | 3.39 ± 0.11 cC | 3.07 ± 0.06 bC |
| **LY** | 3.97 ± 0.10 aF | 6.81 ± 0.04 aCD | 7.10 ± 0.14 aBC | 7.86 ± 0.06 aA | 7.16 ± 0.07 aB | 6.58 ± 0.08 aDE | 6.28 ± 0.06 aE | 2.73 ± 0.05 aD | 6.91 ± 0.10 aB | 7.86 ± 0.05 aA | 7.58 ± 0.06 aA | 6.79 ± 0.09 aB | 6.12 ± 0.06 aC | 5.89 ± 0.11 aC |

Data are expressed as $\log_{10}$ CFU/mL and $\log_{10}$ CFU/g for brine and olives samples, respectively. Results are reported as average value and standard deviation of three replicates. [a–c] Different superscript letters within the same column indicate significant differences at $p < 0.05$. [A–F] Different superscript letters within the same row indicate significant differences at $p < 0.05$.

Overall, at T0 sampling time, no differences were found among L, LY, and C brine and drupe samples for all the analyzed microbial groups. Regarding mesophilic bacteria, starting from the 7th of fermentation, a decreasing trend was observed in brine samples till 120 days. Contrastingly, in olives, after a significant increase till 60 days, a reduction of the mesophilic population was observed at the end of the fermentation (Table 2). Lactobacilli showed, in inoculated (L and LY) brine and olive samples, an increase in the cell density till 60 days followed by a gradual reduction, reaching, at the end of the fermentation process, values of 6.14 log CFU/mL and 7.11 log CFU/mL in L brine and olives, and of 6.63 log CFU/mL and 7.35 log CFU/mL in LY brine and olives, respectively. Similar behavior was observed in C samples, reaching a final cell density of 4.13 log CFU/mL and 4.00 log CFU/mL in brine and olives, respectively (Table 2). Staphylococci markedly decreased throughout the fermentation and were not detected in L and LY samples from the 60th day, whereas in C samples staphylococci were below the detection limit at 120 and 180 days of fermentation, respectively. Similarly, *Enterobacteriaceae* were not detected in both inoculated olive and brine samples (L and LY) from 90 days of fermentation and in C olives and brines at the end of the fermentation (180 days). Significant differences were also appreciable for the eukaryotic population starting from the 7th day. As expected, the level of the yeast population was quite high in LY samples while a significant decrease was registered in L and C samples during the whole fermentation (Table 2).

### *3.3. Detection of Lactobacilli in Table Olives by qPCR*

The detection of the lactobacilli population was carried out by quantitative PCR (qPCR) on olive samples at T7 (after inoculation of the starter cultures), T90 (during the fermentation process), and T180 (end of the fermentation). The detection threshold was calculated based on standard curves and ranged from a maximum value of 9.3 log CFU/mL and a minimum value of 4.3 log CFU/mL with cycle threshold (Ct) values of 6.08 and 22.71, respectively. According to the obtained slope value (−3.32408), both the coefficient of determination (R2) and the reaction efficiency (E) were 99.9%. Table 3 shows the mean values of both the Ct and the cell density of the lactobacilli, expressed as log CFU/mL, at T7, T90, and T180 sampling times. In detail, at T7 the lactobacilli cell density was 8.10 log CFU/mL and 8.19 log CFU/mL in L and LY samples, respectively. At the same sampling time, the population of endogenous lactobacilli detected in C samples showed a cell density of 3.66 log CFU/mL. At the end of the fermentation process, the lactobacilli population detected in L and LY samples remained quite stable whereas an increase of about 3 log units was evidenced in C samples.

**Table 3.** Average values of Cycle threshold (Ct) and microbial cell density expressed as log CFU/mL of Lactobacilli population detected by qPCR in experimental table olives.

| | Cycle Threshold (Ct) | | | Cell Density log CFU/mL | | |
|---|---|---|---|---|---|---|
| | **T7** | **T90** | **T180** | **T7** | **T90** | **T180** |
| **C** | 24.84 ± 0.05 | 22.69 ± 0.09 | 15.17 ± 0.16 | 3.66 ± 0.00 | 4.61 ± 0.00 | 6.58 ± 0.02 |
| **L** | 10.09 ± 0.21 | 8.75 ± 0.08 | 10.87 ± 0.19 | 8.10 ± 0.07 | 8.50 ± 0.02 | 7.86 ± 0.06 |
| **LY** | 9.79 ± 0.24 | 7.81 ± 0.11 | 11.07 ± 0.09 | 8.19 ± 0.07 | 8.78 ± 0.03 | 7.80 ± 0.03 |

## 4. Discussion

Table olives represent one of the main fermented vegetables produced in the Mediterranean area representing an important economic source for producing countries. However, the fermentation process is still empirical and the application of starter cultures, despite being constantly increasing, is not yet common [26]. The importance of using selected starter cultures to control the fermentation process has long been recognised and validated over time [3,27]. In the context of table olives production, it is well established that the simultaneous inoculation of lactobacilli and yeasts standardizes the brine, allowing the producer to obtain a safe final product with reproducible texture and flavours [28–31].

Among the promising strains used as starter cultures in table olives fermentation, isolates ascribed to the *Lpb. plantarum* species are often used for their ecological versatility and metabolic adaptability to a stressful environment [32]. *Lpb. plantarum* plays a key role in the pH decrease, through the production of organic acids, and in the degradation of bitter compounds, through β-glucosidase activity [18]. Yeasts also play an essential role through the production of proteins and glycoproteins, with antifungal effects, and the synthesis of bioactive and antioxidant compounds, which can preserve the integrity of the final product. In addition, yeasts can enhance the growth of lactic acid bacteria and consequently the production of lactic acid [33]. As reported by De Angelis et al. [30], the combination of *Lpb. plantarum* and *Lpb. pentosus* strains with an indigenous strain of *W. anomalus* allowed the acceleration of the fermentation process of Bella di Cerignola table olives. Similarly, Tufariello and co-workers [31] proposed a sequential inoculation strategy (first yeast followed by bacteria) to improve the sensory properties of table olives and accelerate the fermentation process. According to this strategy, in the present study, the use of *Lpb. plantarum* and *W. anomalus* strains as starter cultures for the fermentation of Nocellara Etnea olives allowed us to aid a fast acidification of the brine, ensuring the microbiological safety of the final product. In particular, a noticeable reduction in pH was evident in the inoculated experimental brines already during the first step of the fermentation process, corroborating the results recently reported by Benitez and co-workers [11]. Moreover, at the end of the fermentation process, based on pH values, inoculated samples satisfied the IOOC recommendation [1,19–21,34]. It is well known that, along with setting the optimal conditions of fermentation, special attention should be paid to the selection of raw materials, which could represent a source of spoilage and pathogenic microorganisms. In this regard, our microbiological data revealed that spoilage bacteria belonging to the *B. cereus*, *C. perfringers*, and *E. coli* species were never detected from the earliest stages of the fermentation process, despite, as reported by several studies, the genus *Clostridium* being commonly present in raw olives and/or at the beginning of the fermentation [6,35]. In addition, foodborne pathogens such as *Salmonella* spp. and *L. monocytogenes* were also never detected in all samples investigated, indicating the good quality of raw materials. Both staphylococci and enterobacteria, detected at the beginning of the process, were significantly reduced in inoculated samples by the pH below 4.4 thanks to the selected starter cultures inoculum. This evidence is in accordance with previously reported data showing that the use of *Lpb. plantarum* strains allowed a complete inhibition of spoilage bacteria in Nocellara Etnea olives [19,20,34]. In addition, in discordance with De Angelis et al. [30], the use of the *W. anomalus* F5.60.5 strain, as a starter, did not compromise the survival of lactobacilli during the fermentation, probably due to the establishment of mechanisms of protocooperation as suggested by Anagnostopoulos and Tsaltas [35,36]. As long demonstrated, strains ascribed to the *Lpb. plantarum* species are able to both adhere and colonize the drupe surface, positively influencing the table olive ecosystem [20] and consequently maintaining high the lactobacilli presence. In the present study, the qPCR assay was applied to quantify the lactobacilli population since considered as a positive biomarker for table olives fermentation. In addition, our study demonstrated that the sample inoculated only with lactobacilli exhibited a marked decrease of spoilage bacteria related to a quick and a more pronounced reduction of pH, which assure the safety and quality of the final product. In line with this evidence, the qPCR analysis allowed us to confirm the relative abundance of the lactobacilli group, showing a constant trend over time. As already noteworthy, a polyphasic approach, with combined culture dependent and independent techniques, is a valid tool to better elucidate the microbial community of several different foods [34,37,38]. Among the different molecular techniques, the qPCR method was extensively applied in food microbiology. However, only Correa-Galeote and co-workers [17] investigated the abundance of the microbial population of table olives through this methodology. In addition, the recA gene was targeted to evaluate the occurrence of *Lbp. plantarum* species [17]. Since the species is not the only representative of the *Lactobacillus* group, this approach could be reductive in representing the lactobacilli

population [3]. Our qPCR data demonstrated the dominance of lactobacilli throughout the fermentation process and their survival on the olive surface till the end of the process, indicating both the stability and the safety of the final product.

In conclusion, the present study highlighted the importance of starter cultures to assure the safety of table olives produced at industrial scale. The application of the quantitative PCR (qPCR) appeared to be a promising culture-independent approach to detect and quantify lactobacilli, as a positive biomarker of table olives fermentation.

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