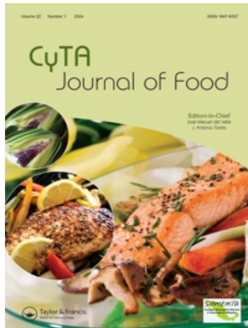

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Enhancing the fermentation of unsalted Moroccan picholine green olives through heat-shock treatment, *Lactiplantibacillus plantarum* S61 inoculation and orange peel addition

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ABSTRACT
Table olives, a vital category among fermented vegetables, come in treated and natural forms. Treated green olives require an alkaline treatment before brining to start fermentation. This study investigates the fermentation of unsalted Moroccan Picholine green olives using a method that includes heat shock treatment, inoculation with Lactiplantibacillus plantarum S61, and orange peel juice. The findings indicate that a heat shock at 70°C significantly reduces enterobacteria and enhances acidification, dropping pH levels from 5.0 to 3.6. In contrast, non-heat-treated olives showed only a slight pH decrease. The method also exhibited high antimicrobial activity, with substantial inhibition of E. coli, P. aeruginosa, L. monocytogenes, and S. aureus. Antifungal activity was noted, with significant inhibition of various strains. These results suggest that combining heat shock, L. plantarum S61, and orange peel juice can significantly improve the fermentation process of green olives, enhancing safety and quality.

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Table olives; heat shock treatment; *lactiplantibacillus plantarum*; orange peel juice; controlled fermentation

1. Introduction

Table olives hold considerable importance as a vegetable staple in regions characterized by a Mediterranean climate. With increasing interest from new markets, their production has had a substantial economic and social impact. Among the leading table olive-producing nations, Morocco has shown a remarkable ability to adapt to the country's pedo-climatic conditions (Fernández et al., 1997).

The olive, classified as a drupe, usually contains a bitter compound known as oleuropein, making the fruit inedible in its raw state (Botta & Cocolin, 2012). Despite the bitterness, the presence of oleuropein in green olives is not harmful to human health (Ghanbari et al., 2012). Various treatments have been applied to reduce their bitterness to render olives suitable for consumption, a factor that often varies based on the region and olive variety (Ercolini et al., 2006). The predominant methods for industrial table olive production include the Spanish-style green olives (or Sevillian) method, which involves the use of NaOH and NaCl (Corsetti et al., 2012; Rejano et al., 2010). Yet, these methods come with drawbacks. Alkaline treatment affects the permeability of olive membranes, thereby elevating the diffusion coefficient of specific nutrients from the fruit to the brine (Boskou et al., 2006; Mettouchi et al., 2016). Also, the elaboration treatment generates the loss of bioactive components and soluble constituents which can have an impact on the color, flavor and texture (Campus et al., 2018; Pozo et al., 2020). These parameters are accountable for the distinctive properties and quality of table olives (Ozdemir et al., 2014). The production process is marked by an extended and unregulated fermentation period, which is linked to a high occurrence of olive spoilage and the generation of significant amounts of wastewater containing phenolic compounds and sodium hydroxide (Ciafardini & Zullo, 2019). This leads to significant environmental challenges (Arroyo-López et al., 2008). The implementation of regulated biological mechanisms can mitigate or alleviate the effects of these drawbacks.

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Lactic acid bacteria (LAB) undoubtedly serve as key contributors to the successful fermentation of table olives (Fernández et al., 1997). Certain strains of lactic acid bacteria have shown the ability to withstand and metabolize oleuropein, the bitter compound found in olives (Rejano et al., 2010). During table olive fermentation, the primary function of lactic acid bacteria is to generate organic acids, notably lactic acid, which lowers the pH and boosts free acidity (Corsetti et al., 2012). In recent decades, the deliberate addition of selected microbial strains has gained importance in table olive production. Inoculation with specific LAB strains allows for better control of the fermentation process (López-García et al., 2022). This lactic acid is pivotal in restraining the proliferation of spoilage and pathogenic bacteria (Abouloifa et al., 2021). The most commonly identified lactic acid bacteria responsible for the fermentation process are mainly of the genus Lactiplantibacillus (López-García et al., 2024). The LAB strain *Lactiplantibacillus plantarum* S61 was selected by the authors for biodegradation capacity of Oleuropein and producing enzymes (β-Glucosidase) responsible for the debittering process of table olives and the generation of lactic acid, resulting in a decrease in the brine's pH (Abouloifa et al., 2020). This approach also facilitates the standardization of product quality and the characteristics of the final product (López-García et al., 2022).

The heat-shock treatment at 80°C enhanced the fermentation profile of unsalted olives by increasing the release of nutrients such as sugars and polyphenols, reducing bitterness (Ghabbour et al., 2023). Numerous studies have documented the advantages of subjecting olives to heat shock before salting, as it improves the fermentation process by eliminating undesirable microbial organisms (Chorianopoulos et al., 2005) and enhancing fruit permeation characteristics (Balatsouras et al., 1983). Furthermore, heat treatment can be employed to enhance quality and prolong shelf life, thus preserving the color. (maintenance of parameter L*) (Abreu et al., 2003; Plotto et al., 2003).

However, the production of orange juice generates enormous quantities of by-product: orange peels (OP). These OP, mainly rich in soluble and insoluble carbohydrates and can serve as raw material. The by-product of orange juice is primarily allocated for animal feed (Bampidis & Robinson, 2006) or for essential oils (Raeissi et al., 2008; Sahraoui et al., 2011) or carotenoids (Aravantinos-Zafiris et al., 1992), erived from peels, among other secondary applications. Prior research has highlighted increased dietary fiber content in orange peel and pomace, along with heightened levels of phenolic compounds and antioxidant capacity, as well as a higher concentration of sugars compared to the fruit itself. These characteristics render it an ideal substrate for fermentation processes (Gorinstein et al., 2001; O'shea et al., 2015).

This study aims to enhance the fermentation of green olives by employing a combination of *Lactiplantibacillus plantarum* S61, a heat shock treatment at 70°C repeated three times for 5 min, and the incorporation of orange peels. The primary goal is to optimize the fermentation process, thereby reducing bitterness, and standardizing the quality of the final product. Additionally, this approach seeks to improve the organoleptic characteristics of fermented olives, leveraging the potential benefits of orange peels as a substrate enriched with phenolic compounds and other beneficial nutrients.

2. Materials and methods

2.1. Preparation of orange peels

In this study, orange peel (OP) was obtained from household waste collected from oranges in Berkane, Morocco. The peels were washed, immersed in distilled water (w/v) and autoclaved at 121°C for 15 min to ensure sterility. After that, they were ground using a sterilized kitchen mixer, and the resulting mixture was filtered through sterilized muslin to obtain OP with a gelling texture. The OPs were subsequently stored at 4°C in sterilized containers until they were needed for further use.

2.2. Inoculum preparation

The *L. plantarum* strain S61 was previously isolated from the traditional fermentation of green olives (Abouloifa et al., 2020). The working stocks of *L. plantarum* S61 were maintained in a glycerol suspension (1/3, v/v) and the strain (2/3, v/v), which were stored at −20°C in Man Rogosa and Sharp (MRS) medium (Oxiod, Milan, Italy). Prior to use, the strain was regularly maintained through subcultures in MRS broth for three consecutive days at 37°C ±1°C for 24 h to obtain 24-h-old strains. The strains were then collected via centrifugation and additionally the cells were suspended in physiological water to achieve a concentration of 10^5 CFU/mL

2.3. Plant material

The green-yellow Moroccan picholine olives, at their ripening stage, were meticulously handpicked during the 2021–2022 season by local farmers from Oujda city. The harvesting process was conducted under optimal conditions with measures in place to prevent the olives from falling onto the ground. Any drop exceeding a height of 1 m would result in critical damage to the fruit. The harvested green olives were of premium quality, characterized by their healthy, fleshy, and firm flesh that exhibited resistance to weak pressure when squeezed between the fingers. The olives were whole, free from deformities, and crushing with a uniform color, without any blemishes except for natural pigmentations. Culling of the batch's wounded or damaged fruits and peduncles ensured that only the highest quality olives were included in the collection.

2.4. Heat treatments

The whole olives underwent a 10-min wash in distilled water, followed by random distribution into each glass bottle for the fermentation process. This involved immersing the olives in a 70°C water bath for 5 min., repeated three times, with the change of water after each heat treatment excepting the last treatment.

2.5. Green olive fermentation

The olives were first subdivided into four 5-l glasses with two repetitions of each glass bottle. Each bottle contained 2 kg of green olives and 2 l of distilled water. The present study

involved the investigation of four different olive fermentation methods. The first essay, not heat treatment and not inoculated (NHT-NI), served as the control and involved neither heat-treated nor inoculated olives. The second essay, heat treatment and not inoculated (HT-NI), was a heat-treated olive fermentation process that entailed the olives underwent immersion in a water bath maintained at 70°C for 5 min, a procedure that was repeated three times, with the water being replaced after each heat treatment. The third essay, heat treatment and inoculated (HT-I), involved the same heat treatment process as (HT-NI) but with an inoculation of 2% (v/v) lactic acid bacteria (20 mL of *L. plantarum* S61 with a concentration of 10^5 CFU/mL). Finally, the fourth essay, heat treatment-orange peel juice and inoculated (HT. OP-I), was a heat-treated and inoculated as in HT-I, but with additional supplementation of 1% orange pulp juice.

All olive fermentation experiments were conducted in duplicate and were consistently maintained at 30°C over the 52-day fermentation period. Water samples were collected under aseptic conditions at different fermentation stages and subjected to comprehensive analyses encompassing both physicochemical and microbiological assessments. This facilitated the identification and quantification of any alterations in the physical, chemical, and microbiological attributes of the water samples, corresponding to each of the four fermentation methods under scrutiny.

2.6. Samples analysis

All trials were incubated in the laboratory at 30°C to maintain optimal fermentation conditions for the lactic acid bacteria. During the fermentation period (52 days), physicochemical analyses were performed regularly on days 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, and 52. Samples were aseptically collected for each analysis. All analyses were repeated in triplicate for statistical analysis.

2.6.1. pH and titratable acidity

The pH was determined using a pH-meter (VWR Symphony SB70P), while the titratable acidity of brine samples was assessed by titrating 10 mL of brine with a 0.1 N sodium hydroxide (NaOH) solution, with phenolphthalein serving as a colored indicator. The outcomes were expressed as a percentage (% w/v) of lactic acid (Abouloifa et al., 2024).

2.6.2. Electrical conductivity

Electrical conductivity was determined utilizing an electrical conductivity meter (CON 2700 conductivity/°C/°F meter).

2.6.3. Surface tension

The surface tension was measured by using a Tensiometer (GIBERTINI Digital Tensiometer TSD), were conducted using the Wilhelmy plate method (Manousakis & Avranas, 2013; Schuster et al., 2014). Surface tension measurements of the brine samples were conducted using the CFS. All the assays were performed in duplicate at 25°C. The surface tension is the energy required to create a square meter of surface. It is denoted in units of $N.m^{-1}$, which corresponds to $J.m^{-2}$ or dyne/cm (Anthony et al., 2019).

2.6.4. Dosage of total sugars

The Olive fermentation water samples from different trials, before and after (4, 8, 12, 28, 36, 44, 48, and 52) days, were assayed for their total sugar content, was determined using the method developed by (Michel et al., 1956). This method relies on the distinctive chemical characteristics of olives. It involves the hot dehydration of hydroxyl groups within an acidic environment, resulting in the formation of furfurals and water molecules. In test tubes containing 2 mL of brine, 0.5 mL of a 5% phenol solution is added while stirring, followed by the immediate addition of 2 mL of concentrated sulfuric acid (97%) with continuous stirring. The reaction is allowed to proceed for 10 min before stirring again, and the tubes are then placed in a water bath at 25–30°C for 20 min until a yellow-red color appears, then the reaction is stopped by a stream of cold water. Absorbance is measured using a visible spectrophotometer (VIS-7220 G/UV-9200) at 490 nm. The absorbance values were transformed into sugar concentrations using a standard range prepared according to the same protocol used for the samples, using glucose as sugar.

2.6.5. Microbiological analysis

Microbiological analysis of olive fermentation water samples included enumeration of total aerobic mesophilic count (TAMC) on Plate Count Agar (PCA) medium, after 72 h of incubation at 30°C; total coliforms (TC) on Deoxycholate Lactose agar (DCL) medium, after 24 h of incubation at 30°C (red colonies); yeasts and molds: on Potato-Dextrose-Agar (PDA) medium, after 48–72 h of incubation at 27°C and lactic acid bacteria (LAB) on de Man Rogosa and Sharpe agar (MRS) medium, supplemented with cycloheximide (0.01%, *w/v*) after 48–72 h incubation at 30°C (round or lenticular colonies).

2.6.6. Antioxidant activity

The antioxidant activity of the brine and ethanolic olive extract samples was evaluated by measuring their ability to scavenge DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals, using the method described by (Li et al., 2012). To assess antioxidant activity, 1 mL of brine obtained by centrifugation at 8000 × g for 10 min was mixed with 2 mL of ethanolic DPPH solution (0.05 mM) from Sigma-Aldrich. The mixture was vigorously mixed and then left to incubate at room temperature in the dark for 30 min. Controls included DPPH and a solution of distilled water, while ethanol mixed with brine supernatant served as a blank. The optical density (OD) of the resulting solutions was measured at 517 nm. Antioxidant activity (AO, %) was calculated using the following equation and determined in triplicate for all assays.

$$AO\% = [1 - (ODsample - ODblank)/ODcontrol]x100$$

2.7. Antimicrobial activity of water samples

The water samples of each studied trials were examined for their antimicrobial activity against Gram-negative bacteria (*Escherichia coli 10,536* and *Pseudomonas aeruginosa* ATCC15442) and Gram-positive bacteria (*Staphylococcus aureus* ATCC6538 and *Listeria monocytogenes* ATCC19117) inoculated in MH broth in the dark at 37°C; three molds: *Geotrichum candidum, Penicillium digitatum P22*, and *Aspergillus niger ASP2*, was maintained in PDA medium in the dark at 25°C, and three yeasts: *Rhodotorula glutinis* ON209167.1, *Candida albicans, Candida pelliculosa* and *Saccharomyces cerevisiae*). The culture was incubated in

yeast extract glucose broth (YEG) from Biokar, France, in the dark at 25°C.

The antimicrobial activity was evaluated using the agar diffusion technique on Muller – Hinton Agar (MHA) plates (Biokar, France) with 6 mm diameter wells, following the method described by (Alsarhan et al., 2013). Fifteen milliliters of supercooled Mueller–Hinton agar medium were poured into Petri dishes and left to solidify and dry at room temperature. Bacterial concentration was adjusted to 10^6 cells/mL, 0.1 mL of the standardized inoculum solution (turbidity 0.5 McFarland barium sulfate standard) for yeast and bacteria, and 10^5 spores/mL for molds, were poured into each dish, and then evenly distributed. Plates were then left to dry for 30 min, 100 µL of brine (NHT.NI, HT.NI, HT.I, HT-OP.I) per well for test, 100 µL of Distilled water for the negative control and 100 µL of Gentamycin for the positive control for antibacterial activity and Cycloheximide for antifungal activity. Petri dishes remained at 4°C for 15–30 min then are incubated in an oven 18 h. The estimate of the antibacterial activity is done by measuring the clear zones (halos) in mm which form around the wells using a caliper. A product is considered active if the diameter of the inhibition zone is greater than 8 mm (Ela et al., 1996).

2.8. Color analysis of fermented olive

Colorimetric measurements on olive fruits were conducted using a Chroma Meters Measuring Head Model CR-400, equipped with computer software for calculating the CIE L* (lightness), a* (redness/greenness), and b* (yellowness/blueness) values. These parameters were measured by scanning an 8 mm surface area of eight olive fruits. To minimize interference from stray light, samples were covered with a box featuring a matte black interior.

2.9. Texture analysis of fermented olive

The hardness was evaluated using a Brookfield texture analyzer, which applies a precisely controlled force to measure the material's resistance. The device utilizes a force sensor to gauge resistance, providing insights into the texture and physical properties of the material. It offers a range of physical measurements, including force, distance, and speed, with high accuracy. The analyzer operates in vertical compression mode, with the probe moving at a consistent speed onto the olive, securely positioned on a base table within an oven.

2.10. Sensorial evaluation of fermented olive

The sensory assessment was performed by a tasting jury made up 25 people (15 women and 10 men of different ages 26–44 years) recruited from students and staff members of the Department of Bioresources, Biotechnology, Ethnopharmacology and health laboratory, Mohammed I University, Oujda. The four-table olive fermentation liquid was coded as follows: A: NHT-NI, B: HT-NI, C: HT-I and D: HT. OP-I.

Processed olives were evaluated using a descriptive test in which the main quality attributes of table olives were presented, using a scale of 1 to 9: (1, 2 and 3 poor; 4, 5 and 6 average; 7, 8, and 9 good). The quality attributes were categorized into four groups, corresponding to olfactory sensations (smell, flavor), taste attributes (salinity, acidity, bitterness, and astringency), kinesthetic sensations (Texture) (Aponte et al., 2010). Five olives from each sample are tasted to ensure uniformity.

2.11. Data analysis

All tests were performed in duplicate, and the results are presented as the mean ± standard deviation. A one-way ANOVA was conducted to compare all the means, with significance set at $p < .05$. Additionally, the Student-Newman-Keuls (S-N-K) comparison test was used. The analyses were carried out using GraphPad Prism 8 software (San Diego, California, USA).

3. Results and discussion

3.1. pH and acidity

Whatever, the applied heat shock, after 2 days of fermentation, the pH values (Figure 1(a)) of the olive fermentation liquid show a significant reduction from 5.0 ± 0.04 to 3.6 ± 0.03 (for HT.NI, HT.I and HT.OP-I) but in the control olives (NHT.NI) the pH values show a slight decrease from 5.30 ± 0.04 to 4.86 ± 0.03. These values fluctuated progressively to reach stability by the end of fermentation, approximately around 4.10 for all trials. These pH values are similar to those observed by (Angelis et al., 2015; Caponio et al., 2019). The final pH lower than 4.5 could allow a good stability of the fermented product (Antonio et al., 2008).

The titratable acidity evolved in the opposite direction of the pH (Figure 1(b)). The values of titratable acidity obtained showed an increase during fermentation for all the trials to reach maxima of 0.87 ± 0.029% for the HT.NI trials, and 0.89 ± 0.007% for the HT.I trials, and 0.92 ± 0.029% for the HT.OP-I, while for the NHT.NI trial did not exceed the value of 0.78 ± 0.007%. Significant differences ($p < .05$) were noted in pH and free acidity values among the experiments, which were attributed to the effects of heat shock, orange peel, and inoculation. The increase in acidity is mainly due to the fermentation of free and/or Oleuropein-bound sugars into organic acids mainly by lactic acid bacteria and yeasts (Marsilio et al., 2008). The product obtained is stable after the end of fermentation.

3.2. Electrical conductivity

Electrical conductivity (EC) is a critical parameter in monitoring the fermentation process of olives, serving as an indicator of the ionic strength of the fermentation brine, reflecting the concentration of ions released from the olives and the metabolic activities of microorganisms. As depicted in Figure 1(c), the EC of the fermentation brine exhibited a general increasing trend over time before stabilizing during the ripening phase of the olives. Specifically, for NHT.NI, EC stabilized after 20 days, reaching a value of 6.09 ± 0.004 mS/cm; HT.NI and HT.I stabilized after 16 days, with EC values of 6.29 ± 0.04 mS/cm and 6.13 ± 0.07 mS/cm, respectively; and HT-OP.I showed stabilization at 12 days, with an EC value of 6.08 ± 0.009 mS/cm. These findings align with those of (Lirola, 1992) who observed similar trends in the fermentation of tropical fruits. The initial increase in EC can

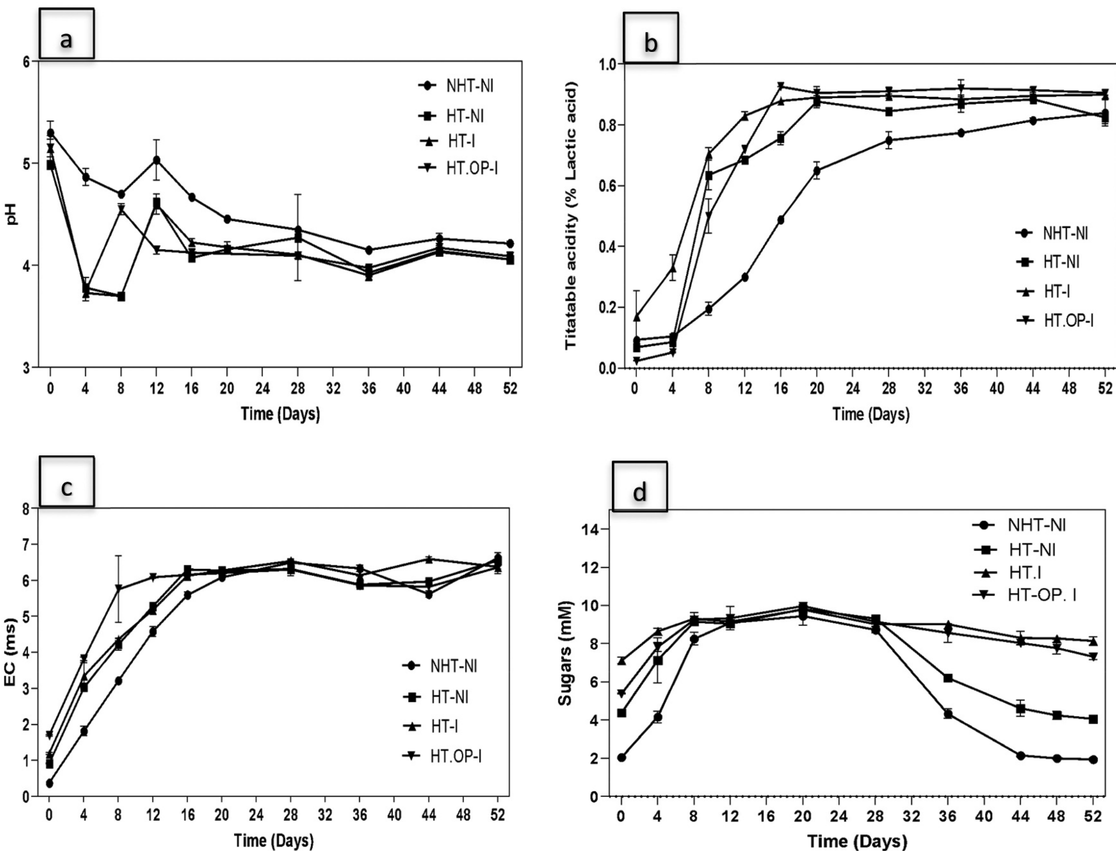

Figure 1. The pH (a), free acidity (%) (b), total sugars (mM) (c) and electrical conductivity (d) evolution during controlled fermentation of NHT-NI, HT-NI, HT-I, and HT.OP-I is depicted. Data represent the mean of six measurements, and standard error is indicated on the bars.

be attributed to the release of ions from the olives and the metabolic activities of fermentative microorganisms, which produce organic acids and other ionic metabolites. During fermentation, olives release various ions into the brine, and the activity of lactic acid bacteria (LAB) further contributes to the increase in EC by producing organic acids that lower the pH and enhance ion release. These trends suggest that the combination of heat shock, inoculation with *Lactiplantibacillus plantarum* S61, and the addition of orange peel juice effectively enhance the fermentation process, leading to more rapid stabilization of EC and potentially improving the overall quality and consistency of the fermented olives.

3.3. Surface tension

The results of surface tension tests (brine samples) before the fermentation process (Table 1), we notice that highlighting the olive in water, the T.S will decrease compared to water without olive (73 mN/m). So, if we compare between the tests subjected to pasteurization and unpasteurized, we notice a high value of T.S in the unpasteurized tests (NHT.NI = 35.06 mN/m), and if we compare between the tests inoculated and not inoculated with lactic acid bacteria, we note that inoculation will slightly reduce the T.S, with 48.9 mN/m and 47.33 mN/m for HT.NI and HT.I respectively. Thus, the addition of orange peel juice (HT-OP.I) slightly increases the T.S from 47.33 mN/m (HT.I) to 46.8 mN/m (HT-OP.I). These observations suggest that both pasteurization and inoculation notably influence surface tension, whereas the addition of orange peel juice has a more marginal effect. These data could indicate that the physicochemical properties of the brine are significantly altered by the fermentation preprocess treatments, which could have implications for the overall fermentation dynamics and the quality of the final olive product.

3.4. Sugar content

The results of the total sugar content obtained in brine samples during the fermentation process are reported in Figure 1(d). The soluble sugar values exhibited a slight increase up to the 20th day of fermentation, reaching

Table 1. Surface tension before and after fermentation of green olives and variation of the compressive stress σ with the compression ration $\boldsymbol{\varepsilon}$ for different treatment of olive: NHT-NI, HT-NI, HT-I, and HT.OP-I, for a compression speed (V = 1 mm/s). $\boldsymbol{\varepsilon}$* and $\boldsymbol{\sigma}$* denote the breaking strain rate and the breaking stress, respectively.

Brine	Surface tension (mN/m) Day 0	Surface tension (mN/m) Day 52	ΔST (mN/m)	*E* (kPa)	ε_c (%)	ε* (%)	σ* (kPa)
NHT-NI	$53.06^{a} \pm 0.6$	$47.27^{a} \pm 0.2$	−5.79	43	0.9	14.86	350.93
HT-NI	$48.9^{ab} \pm 0.2$	$46.67^{c} \pm 0.3$	−2.23	5.88	2.2	14.96	129.01
HT-I	$47.33^{b} \pm 0.2$	$46.13^{b} \pm 0.6$	−1.2	4	1.9	14.97	119.01
HT-OP-I	$46.8^{b} \pm 0.1$	$46.43^{b} \pm 0.2$	−0.37	4.8	1.3	14.95	116.45

[a]Means on the same column of each brine with different lowercase letters differed significantly ($p < .05$).

a range of 2.046 mM ±0.13 to 9.314 mM ±0.31 in all assays. This initial rise can be attributed to the slower diffusion rate of sugars from the olive pulp to the brine compared to their consumption by the microorganisms. The increase in permeability of the olive skin due to the action of microbial cellulases and pectinase accelerates the efflux of sugars. The microorganisms enter the stationary phase, which lowers their need for sugars used in multiplication, allowing the sugars to reach maximum values of between 9–10 mM in all trials. The presence of heat shock, orange peel juice, and inoculation with *L. plantarum* S61 had a significant impact on the accumulation of sugar content in the water of the olives ($p < .05$). Our results showed that the mean total sugar value significantly increased with the heat shock treatment at 70°C (2–4 mM), compared to that *of L. plantarum* S61 (5 mM) and orange peel juice (7 mM). These values exhibited a steady decrease until the conclusion of the fermentation process, reaching 1.940 mM ±0.07 in NHT.NI olives, 4.05 mM ± 0.7 in HT.NI, 7.32 mM ± 0.15, and less than 8.15 mM ±0.21 in HT-OP.I olives. These findings are consistent with previous studies. For instance (Balatsouras et al., 1983), observed a significant impact of heat shock on sugar accumulation, while (Argyri et al., 2014) reported a steady decrease in sugar content during fermentation.

These findings highlight the intricate relationship between microbial activity, enzymatic breakdown of olive tissues, and sugar metabolism throughout the fermentation process. This complex interplay is crucial for understanding how sugars are released and utilized within the olive brine, impacting the quality and consistency of the final product. Further research should investigate the specific enzymatic pathways and microbial interactions that govern these sugar dynamics. By focusing on these areas, it may be possible to refine and optimize fermentation processes, ultimately enhancing the quality and consistency of fermented olives. This could lead to more controlled fermentation environments and potentially new methods to manipulate the biochemical processes for improved flavor and nutritional profiles in table olives.

3.5. Microbiological analysis

During the initial 2 weeks of the process, all experiments showed a rapid increase in the microbiota associated with olives.

3.5.1. Lactic acid bacteria (LAB)

The results of the microbial population of LAB in olive experiments are presented in Figure 2(a). In NHT.NI olives, The native LAB population showed an initial increase from 3 Log CFU/mL to about 6 Log CFU/mL within the initial 16 days, followed by a minor decrease, stabilizing around 5–4.5 Log CFU/mL towards the conclusion of the fermentation process.

In olives subjected to HT.NI, the indigenous LAB population initially increased from 3 Log CFU/mL to 7 Log CFU/mL within the first 16 days. This was followed by a slight decrease, stabilizing at around 6 Log CFU/mL by the end of fermentation. A notable distinction ($p < .05$) was detected in LAB biomass, ascribed to the impact of heat treatment at 70°C, which markedly increased the LAB content compared to that of NHT.NI olives.

In olives subjected to HT.I, the LAB population exhibited notable growth within the initial 16 days of fermentation, rising from 5 Log CFU/mL to approximately 9 Log CFU/mL. Subsequently, there was a slight decline to 8 Log CFU/mL by the end of the fermentation period. Significant differences were observed when comparing olives HT.I to those without HT.NI. The interaction between inoculation effects significantly influenced ($p < .001$) the evolution of the LAB population.

The green olives were subjected to a series of heat treatments at 70°C, repeated three times for 5 min each. Subsequently, they were HT-OP.I. In these samples, the LAB population exhibited significant growth, reaching approximately 9 Log CFU/mL by the 16th day of the process after starting at 5 Log CFU/mL. Thereafter, it experienced a slight decrease to 8.5 Log CFU/mL until the process's conclusion. The initial stages of the fermentation process were marked by the dominance of LAB, facilitated by the heat treatment. This treatment provided abundant nutrients, including sugars, polyphenols, and vitamins. Significant differences ($p < .05$) in LAB counts were observed when heat treatment, inoculation and/or orange peel juice addition were applied, compared to uninoculated olives. This finding is consistent with previous reports by (Panagou et al., 2003). The decrease in LAB population could be attributed to the elimination of LAB species that are not tolerant to high acidity levels. Additionally, it may also be influenced by the presence of polyphenols in the fermentation liquid (Landete et al., 2008; Rodriguez et al., 2009). In accordance with (Panagou et al., 2003), The final concentration of LAB is crucial for shaping the functional characteristics of the final product, as this microbial population plays a key role in determining the flavor and texture of the end products (Fernández et al., 1997; Sánchez et al., 2000).

3.5.2. Yeasts and molds

The microbial population findings of yeasts and molds in olive experiments are depicted in Figure 2(b). Initially, this population ranged from 3 to 3.5 Log CFU/mL on the first day, and in all experimental groups, there was a rapid development after 4 days of fermentation, reaching a maximum of 8–8.5 Log CFU/mL. This rapid development of yeasts and molds may be attributed to the accumulation of nutrients and spoilage microorganisms present in fermented olives (Arroyo-López et al., 2008, 2012; Sidari et al., 2019). After this phase, the yeast population exhibited a slight decrease, stabilizing at around 7–8.2 Log CFU/mL, 8 Log CFU/mL, 6.5–7 Log CFU/mL, and 5.5–5 Log CFU/mL for NHT.NI, HT.NI, HT.I, and HT-OP.I respectively until the end of the process. Similar results have been reported by (Chorianopoulos et al., 2005) The growth rate is primarily dependent on the concentration of the carbon source rather than the type. The heat shock treatment resulted in a significant decrease ($p < .05$) in the population of yeasts and molds. Slight variations in evolution were observed in inoculated olives and those with orange peel juice addition compared to the uninoculated ones.

3.5.3. Total aerobic mesophilic flora

At the start of fermentation, the population of the TAMF (Figure 2(c)) already present in the olives (fruit, water, etc.) was observed on the first day of fermentation. The total aerobic mesophilic flora (TAMF) rapidly increases in all trials,

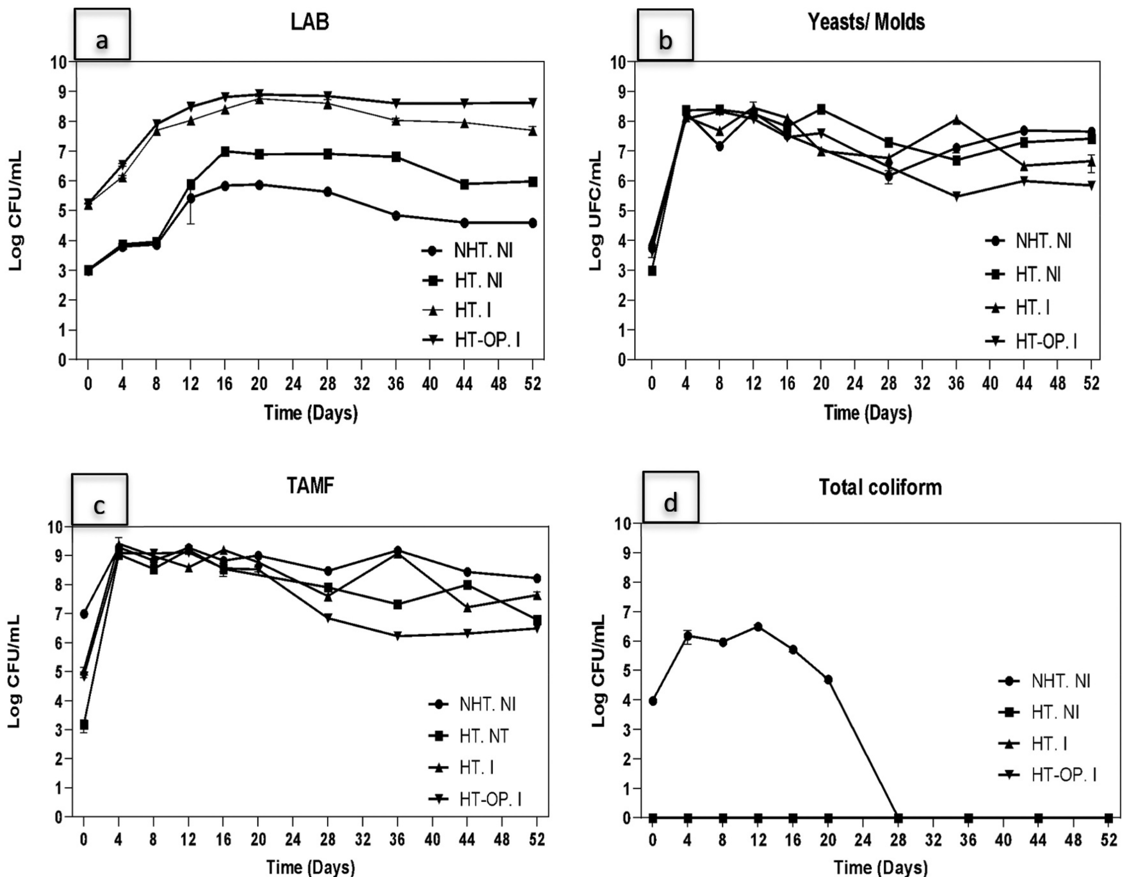

Figure 2. Evolution of microbial groups (TAMF (a), total coliforms (b), LAB (c) and Yeasts/Molds (d)) during the controlled fermentation of: NHT-NI, HT-NI, HT-I, and HT.OP-I. The data represent the mean of six measurements, and the standard error is displayed on the bars.

reaching its peak after 4 days, approximately at 9 log CFU/mL for all trials. Subsequently, there is a slight decrease, and the population remains fluctuating until the end of the fermentation process. Comparing the heat-treated and the non-heat-treated trials, we notice that the non-heat-treated trial is relatively more charged than the heat-treated one.

Comparing the inoculated and non-inoculated treatments, the inoculated trial still shows a certain delay in the development of TAMF, and also the addition of orange juice decreases the microbial development. This could be due to the effect of washing which eliminates a large part of the natural microbiota associated with the olives, as well as fermentable nutrients. These results are similar to those reported by (Rokni et al., 2015).

3.5.4. Coliforms

The results of total coliforms are presented in Figure 2(d) Coliforms were entirely absent in the treatments subjected to a heat treatment of 70°C (HT.NI, HT.I, and HT-OP.I) from the start of fermentation. In contrast, in NHT.NI, coliforms were present and only disappeared after 28 days of fermentation. The coliform population was evaluated only in non-heat treated olives; from the 28th day onward were found to be below the detection threshold, confirming the favorable progression of the olive fermentation process towards lactic fermentation (Rokni et al., 2015).

3.6. Antioxidant analysis

In all four treatments, the methanolic olive extracts showed significant DPPH radical scavenging activities. The lowest IC50 (Figure 3) was represented by the treatments not subjected to heat treatment, 0.463 ± 0.032 µl/mL suggesting a high antioxidant capacity to neutralize free radicals. Therefore, we can conclude that the treatment reduced the molecules which play an antioxidant role. The results indicate that the untreated methanolic olive extracts exhibit the highest antioxidant capacity, as evidenced by the lowest IC50 value. This suggests that the antioxidant molecules, likely polyphenols and flavonoids, are more prevalent or active in the untreated samples. Heat treatment, while beneficial for reducing microbial load and enhancing the safety of fermented olives, appears to diminish the antioxidant properties. This could be due to the degradation of heat-sensitive antioxidant compounds during the heat shock process. Comparing our findings with existing literature, we observe a similar trend where thermal processes reduce the antioxidant activities of plant-based foods (e.g., Gorinstein et al., 2001). This aligns with the understanding that phenolic compounds, which are potent antioxidants, can degrade under high temperatures (Ciafardini & Zullo, 2019). However, the trade-off between microbial safety and antioxidant retention needs careful consideration. The inoculation with *L. plantarum* S61 did not significantly alter the antioxidant activity, suggesting that microbial fermentation primarily impacts other biochemical pathways without substantially affecting the antioxidant compounds.

3.7. Antimicrobial activity

The findings regarding the antibacterial activity of brine samples are outlined in Table 2. Concerning *E. coli* a slight

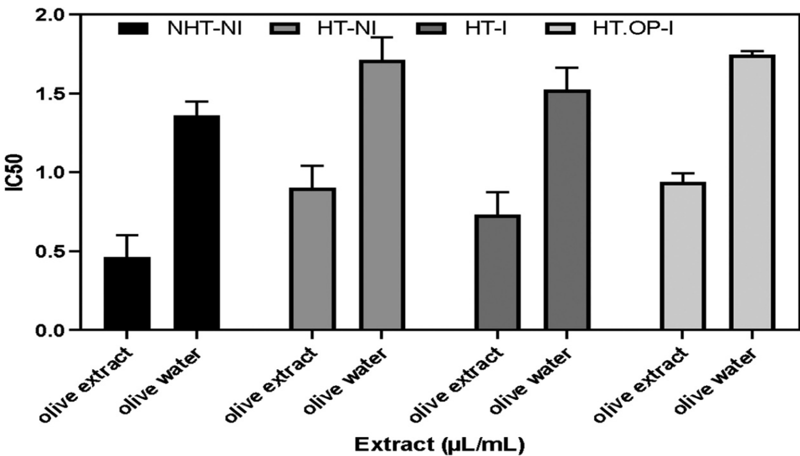

Figure 3. Antioxidant activity (IC50) of olive brine and methanolic extracts of fermented olive. NHT-NI, HT-NI, HT-I, and HT.OP-I. The data represent the mean of six measurements, and the standard error is displayed on the bars.

Table 2. Antibacterial and antifungal activity of brine inhibition zone in mm of: NHT-NI, HT-NI, HT-I, and HT.OP-I.

	Inhibition zone in mm			
	Gram-negative bacteria		Gram-positive bacteria	
Brine	*Escherichia coli*	*Pseudomonas aeruginosa*	*Listeria monocytogenes*	*Staphylococcus aureus*
NHT-NI	10.0^{aD} ±0.1	09.0^{aC} ± 0.1	10.7^{aD} ± 0.6	09.7^{aD} ± 0.6
HT-NI	10.5^{cD} ± 0.5	09.5^{dC} ± 0.5	11.3^{bC} ± 1.2	15.3^{aC} ± 0.6
HT-I	13.5^{bC} ± 0.5	09.5^{dC} ± 0.5	10.3^{cD} ± 0.6	14.7^{aD} ± 0.6
HT.OP-I	14.5^{cB} ± 0.5	13.5^{dB} ± 0.5	19.3^{bB} ± 0.6	20.0^{aB} ± 1.0
Gentamicin	31.0^{aA} ± 1.0	21.0^{cA} ± 1.7	30.3^{bA} ± 0.6	30.7^{bA} ± 1.2

	Geotrichum candidum	*Penicillium digitatum*	*Aspergilus niger*	*Rhodotorula glutinis*	*Candida albicans*	*Saccharomyces cerevisiae*
NHT-NI	ND	ND	ND	21.0^{aD} ± 1.0	ND	14.3^{bD} ± 0.6
HT-NI	ND	ND	ND	22.2^{aC} ± 1.9	ND	22.5^{aB} ± 0.5
HT-I	ND	ND	ND	21.5^{aD} ± 1.0	ND	17.5^{bC} ± 0.5
HT.OP-I	ND	ND	ND	23.0^{aB} ± 1.0	ND	22.5^{bB} ± 0.5
Cycloheximide	35.0^{a} ± 1.0	30.0^{b} ± 1.0	22.0^{e} ± 1.0	28.0^{cA} ± 1.0	21.0^{f} ± 1.0	27.0^{dA} ± 1.0

Note: Values are mean ± standard error of triplicates.

[a]Means in the same line of each strain with different capital letters differed significantly ($p < .05$).

[b]Means on the same column of each brine with different lowercase letters differed significantly ($p < .05$).

inhibition was highlighted for the two treatments NHT.NI (10 mm) and HT.NI (10 mm) with no significant difference. While for HT.I represents a inhibition diameter of 13.5 mm, and the mesh diameter inhibition is 14.5 mm for HT-OP.I with a significant difference ($p < .5$).

And for *P. aureginosa* it has very low inhibition for the three treatments NHT.NI, HT.NI and HT.I with 9 mm, 9.5 mm and 9.5 mm in diameter inhibition, respectively without significant difference. While for the HT-OP.I treatment represents a good inhibition of 13.5 mm diameter with a significant difference with the others ($p < .5$). And, for the two gram-positive bacteria (*L. monocytogenes* and *S. aureus*), the HT-OPW.I treatments represent a very significant inhibition of 19.3 mm for *Listeria monocytogenes* and 20 mm for Staphylococcus aureus, then the HT.NI with 13.3 mm for *L. monocytogene* and 15.3 mm for *S. aureus*, and last there are the two treatments NHT.NI and HT.I with 9.7 mm and 14.7 mm for *S.aureus*, 10.7 mm and 10.3 mm for *L. monocytogene* respectively.

Table 2, shows the antifungal activity of brine, measured by the inhibition zone in millimeters, against different fungi for various treatments of olives: NHT-NI, HT-NI, HT-I, and HT. OP-I. Cycloheximide, a known antifungal agent, is used as a control. *Geotrichum candidum, Aspergillus niger* and *Penicillium digitatum*: No antifungal activity detected (ND) in any brine treatments. For *Rhodotorula glutinis*: NHT-NI: 21.0 mm, HT-NI: 22.2 mm, HT-I: 21.5 mm, HT.OP-I: 23.0 mm. The highest antifungal activity is observed with HT.OP-I. *Candida albicans*: No antifungal activity detected (ND) in any brine treatments. *Saccharomyces cerevisiae*: NHT-NI: 14.3 mm, HT-NI: 22.5 mm, HT-I: 17.5 mm, HT.OP-I: 22.5 mm. The highest antifungal activity is observed with HT-NI and HT.OP-I. Heat treatment and inoculation generally increase the antifungal activity of the brine. The combination of heat treatment and orange peel juice inoculation (HT.OP-I) shows the highest antifungal activity against Rhodotorula glutinis. Treatments involving heat treatment and inoculation show significant improvements in antifungal activity compared to the control (NHT-NI). These results suggest that combining heat treatment and inoculation with specific microbial strains and orange peel juice can enhance the antifungal properties of brine used in olive fermentation.

The inhibition diameters depend on the dose deposited on the disc. Olives contain numerous compounds with antimicrobial action, these constituents include phenolic compounds and flavonoids (Rojas et al., 1992), the antimicrobial power of the extracts depends on their chemical compositions. According to (Cowan, 1999), the impact of extracts on microorganisms is attributed to various classes of polyphenols, particularly tannins and flavonoids. This effect is contingent on the location and quantity of hydroxyl groups present on the phenolic compound. Moreover, it is evident

that higher hydroxylation results in increased lightness, which explains the heightened activity observed in the fourth treatment: HT-OPW.I.

3.7.1. Color parameters determination

The color attribute is another crucial factor influencing the acceptance of a food product. Since fresh olives have been subjected to heat treatment (pasteurization) and/or microbial activity (inoculation of lactic acid bacteria) and added with orange peel juice before undergoing fermentation the impact of these treatments on color and texture was evaluated.

The mean values of CIE l*a*b* parameters of olive color are presented in Tables 3 and 4. Across all experiments, the olives exhibited high lightness (L) within the range of 43–52, typically indicative of favorable color. The rise in olive temperature caused by heat shock increased lightness (L*) and yellowness (b*), while reducing greenness (a*). Among the experiments, NHT.NI showed the lowest mean values of L* (48.7), b* (27.1), and a* (4.8). HT.NI led to a notable rise in the mean values of lightness (L* = 31.4) and yellowness (b* = 34.2), along with a decrease in greenness (a* = −1.1). In contrast, inoculation with *L. plantarum* S61 led to a notable increase in mean lightness values (L* = 47.3) and a reduction in greenness (a* = −0.25), while maintaining a relatively consistent level of yellowness (b* = 28.67) (see Table 2). Additionally, subjecting the olives to heat shock at 70°C, along with inoculation of *L. plantarum* S61 and addition of orange peel, resulted in a significant elevation in mean lightness (L* = 49.5) and yellowness (b* = 31.1), coupled with a decrease in greenness (a* = −0.8).

Inoculation enhanced all CIE Lab* parameters, with the most significant color improvement observed in olives that received inoculation with *L. plantarum* S61 and were supplemented with orange peel (Minguez-Mosquera et al., 1994; Ramírez et al., 2016).

3.8. Texture

Texture holds considerable importance in consumer acceptance of a product and is often regarded as the most important property in many cases (Luckett, 2016). Each sample consisted of a batch of 10 olives from each jar, meticulously chosen to avoid physical deformities and/or mechanical damage, thus ensuring uniformity in size. Each batch sample was directly taken from the jar and horizontally placed, centered under the probe before measurement. All analyses were conducted at room temperature. Parameters measured included elastic modulus (E), critical strain, strain at failure (e*), and stress at failure (s*) during uniaxial compression of various olive samples (Table 1).

It was observed that the values after fermentation of the NHT.NI treatment were significantly higher. This could be explained by the absence of heat treatment. However, in the other trials, there were no significant differences observed, suggesting that neither inoculation nor the addition of orange peel juice impacted the texture profile of the final product (Anagnostopoulos et al., 2019). Texture loss is heavily influenced by the enzymatic activity of the dominant microbial population and, in certain instances, can lead to softening as a result of the degradation of pectin substances in the cell wall and middle lamella (Fernández-Bolaños et al., 2002). The latter depends on critical conditions of the brine, such as pH (Fadda et al., 2014).

Table 3. Average values and mean squares for the analysis of variance regarding olive color using the CIE lab* parameters (**L*** = lightness, **a*** = greenness, **b*** = yellowness) throughout the duration of controlled fermentation. NHT-NI, HT-NI, HT-I, and HT.OPW-I.

	CIE L*a*b* parameters								
	L*			a*			b*		
	Whole olive								
Brine	0 h	2 h	4 h	0 h	2 h	4 h	0 h	2 h	4 h
NHT-NI	48.7D ± 2.9	45.2C ± 1.6	43.8D ± 2.3	2.8A ±0.6	3.6A ±0.5	4.2A ±0.9	27.1D ± 2.0	22.3E ± 1.1	20.3E ± 1.5
HT-NI	51.4B ± 2.2	44.9D ± 0.9	44.7C ± 0.6	−1.1C ± 0.4	0.2B ± 0.3	0.6C ± 0.3	34.2A ±3.8	27.2C ± 0.5	26.4C ± 0.4
HT-I	47.3E ± 0.6	45.04C ± 0.4	44.86C ± 1.6	−0.25B ± 0.3	0.24B ± 0.1	0.84B ± 0.2	28.67C ± 0.9	24.2D ± 0.2	24.05D ± 0.7
HT.OP-I	49.5C ± 1.9	49.4B ± 2.2	48.7B ± 2.3	−0.8C ± 0.5	0.1C ± 0.5	0.3D ± 0.5	31.1B ± 3.4	28.3B ± 1.7	27.1B ± 1.8
Fresh olive	52.2A ±0.4	52.0A ±0.4	49.4A ±0.3	−12.7D ± 0.2	−11.5D ± 0.4	−11.3E ± 0.2	31.3B ± 0.1	30.5A ±0.2	30.1A ±0.2
	Pitted olive								
	0 h	2 h	4 h	0 h	2 h	4 h	0 h	2 h	4 h
NHT-NI	$51.61^{C} \pm 1.5$	$41.00^{C} \pm 1.6$	$39.20^{B} \pm 0.2$	$2.11^{A} \pm 0.2$	$6.02^{A} \pm 1.5$	$6.62^{A} \pm 0.2$	$22.62^{C} \pm 1.5$	$15.79^{D} \pm 1.6$	$15.09^{D} \pm 0.4$
HT-NI	$51.57^{C} \pm 0.4$	$42.86^{B} \pm 0.2$	$41.22^{A} \pm 0.2$	$0.76^{D} \pm 0.1$	$5.10^{B} \pm 2$	$5.96^{B} \pm 3.6$	$24.99^{B} \pm 0.7$	$22.93^{A} \pm 0.6$	$21.03^{A} \pm 1.2$
HT-I	$49.16^{D} \pm 0.7$	$41.67^{C} \pm 0.2$	$39.82^{B} \pm 0.1$	$1.11^{B} \pm 0.5$	$4.49^{C} \pm 0.8$	$5.10^{B} \pm 0.9$	$21.43^{D} \pm 0.5$	$17.44^{C} \pm 0.2$	$16.89^{C} \pm 0.1$
HT-OP-I	$52.38^{B} \pm 0.7$	$42.82^{B} \pm 0.6$	$39.54^{B} \pm 0.4$	$0.87^{C} \pm 0.3$	$5.52^{B} \pm 0.9$	$5.74^{B} \pm 0.2$	$26.38^{A} \pm 0.4$	$22.48^{A} \pm 0.4$	$21.44^{A} \pm 0.2$
Fresh olive	$53.71^{A} \pm 1.7$	$44.89^{A} \pm 1$	$41.96^{A} \pm 0.1$	$-5.47^{E} \pm 0.5$	$-1.51^{D} \pm 3.8$	$-1.30^{C} \pm 0.1$	$22.81^{C} \pm 0.7$	$20.03^{B} \pm 0.6$	$20.00^{B} \pm 0.5$

[A-E]Means on the same column of each brine with different lowercase letters differed significantly ($p < .05$).

Table 4. Mean values and mean squares (Table 4) of analysis of variance for olive color based on the IB (browning index) parameters over time and *ΔE** (total color difference) after controlled fermentation. NHT-NI, HT-N, HT-I, and HT.OPW-I.

	IB						ΔE*	
	Whole olive			Pitted olive				
Brine	0 h	2 h	4 h	0 h	2 h	4 h	Whole olive	Pitted olive
NHT.NI	$12.5^{A} \pm 1.8$	$14.47^{A} \pm 1$	$15.55^{A} \pm 1.5$	$8.73^{A} \pm 0.2$	$19.45^{A} \pm 1.3$	$21.79^{A} \pm 1.2$	$-2.57^{D} \pm 0.1$	$-8.89^{C} \pm 1.1$
HT.NI	$5.35^{B} \pm 2.1$	$6.50^{C} \pm 0.5$	$8.18^{B} \pm 0.4$	$6.42^{B} \pm 1.3$	$18.12^{B} \pm 1.1$	$20.59^{B} \pm 2.1$	$-1.49^{C} \pm 0.3$	$-7.96^{B} \pm 0.3$
HT.I	$5.17^{B} \pm 0.6$	$7.00^{B} \pm 0.2$	$8.81^{B} \pm 0.8$	$6.75^{B} \pm 0.6$	$15.65^{C} \pm 0.1$	$17.88^{C} \pm 0.2$	$-3.2^{E} \pm 0.2$	$-6.51^{A} \pm 1.3$
HT.OP.I	$5.60^{B} \pm 1.9$	$6.80^{C} \pm 1.4$	$7.95^{C} \pm 1.5$	$6.81^{B} \pm 2.1$	$19.08^{A} \pm 0.3$	$21.01^{A} \pm 0.6$	$-0.92^{B} \pm 0.1$	$-6.95^{A} \pm 1.1$
Fresh olive	$-19.24^{C} \pm 0.2$	$-18.33^{D} \pm 0.3$	$-18.81^{D} \pm 0.2$	$-6.62^{C} \pm 0.4$	$0.86^{D} \pm 1.5$	$1.45^{D} \pm 1.1$	$-0.65^{A} \pm 0.4$	$-7.77^{B} \pm 0.2$

[A-E]Means on the same column of each brine with different lowercase letters differed significantly ($p < .05$).

3.9. Consumer testing

According to the results of the hedonic scale, notable differences ($p < .05$) were observed among all debittering methods regarding overall appearance, flavor, taste, texture, and bitternes. Consumer testing results revealed that the higher overall appearance values found in the HT.I and HT-OP.I tests were significantly followed by HT.NI and NHT.NI respectively.

4. Conclusion

This study demonstrated that heat-shocking Moroccan Picholine green olives at 70°C, followed by inoculation with *Lactiplantibacillus plantarum* S61 and the addition of orange peel juice, significantly enhanced the fermentation profile. The heat treatment increased nutrient release, including sugars and polyphenols, which contributed to reducing bitterness and improving the color of the fermented olives. Inoculation with *L. plantarum* S61 facilitated a significant reduction in pH and a substantial increase in free acidity, thereby enhancing the antimicrobial activity against various pathogens such as *E. coli*, *P. aeruginosa*, *L. monocytogenes*, and *S. aureus*. The addition of orange peel juice further boosted the antimicrobial inhibition and overall fermentation process, leading to better sensory attributes such as bitterness, color, texture, and acidity, and reduced spoilage in the fermented olives. These findings suggest that the combined method of heat-shocking, inoculation with *L. plantarum* S61, and the addition of orange peel juice is effective for the biological debittering and fermentation of unsalted olives, offering potential antioxidant and antimicrobial benefits. Additionally, this approach promotes environmental protection by eliminating chemical waste discharge, making it a promising technique for producing healthier, salt-free olives.

Disclosure statement

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