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Naturally Fermented Gordal and Manzanilla Green Table Olives: Effect of Single Yeast Starters on Fermentation and Final Characteristics of the Products

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Abstract: The influence of selected yeast starters (*Kluyveromyces lactis* L39, *Kazachstania humilis* AG5, *Nakazawaea molendinolei* IG9, *Candida diddensiae* IG12, and *Candida adriatica* L30) on the fermentation and final characteristics of natural-style Gordal and Manzanilla green table olives was investigated. In both cultivars, the addition of yeast starters had no significant influence on the evolution of physicochemical parameters or the final main metabolites compared to noninoculated olives. In the Gordal cultivar, *K. lactis* L39 originated the greatest enrichment of volatile compounds, whereas *K. lactis* L39 and *C. adriatica* L30 gave the best volatile profiles in the Manzanilla cultivar. In both cultivars, the β-glucosidase-positive strains *N. molendinolei* IG9, *C. diddensiae* IG12, and *C. adriatica* L30 produced no significant decrease in the total phenolic content at the end of fermentation. Although the yeast starters had a significant effect on the volatile contents of the fermented products, they did not have a significant influence on the main sensory characteristics perceived by a sensory panel. A significant linear relationship ($R^2 = 0.815$, $p < 0.001$) was found and validated between the perceived bitterness intensity and the content of total phenols in olive pulp, providing a simple and objective method for the evaluation of bitterness in table olives without the need for sensory analysis.

Keywords: table olives; Manzanilla; Gordal; fermentation; starter; yeasts; volatile; phenolic; sensory

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1. Introduction

Fermented green table olives are among the most popular fermented vegetable products in Western countries, constituting an important component of the Mediterranean diet. The most significant industrial production methods of fermented green table olives are the Spanish-style method, which involves a debittering process with NaOH solution, and the natural-style method where the fruits are brined without going through a NaOH treatment and left to ferment until they lose their bitterness, at least partially [1]. The natural-style green table olives are different from the Spanish-style olives, mainly due to their taste, color, and residual bitterness. The microbial groups involved in natural fermentations are particularly influenced by the characteristics of the olive cultivar [2,3]. Gordal (*Olea europaea Regalis*) and Manzanilla (*Olea europaea pomiformis*) are the two Spanish olive varieties with the most international renown as green table olives due to the fineness of their pulp and their excellent organoleptic characteristics in general. Lactic acid bacteria (LAB) and yeasts mainly promote natural fermentation in the Gordal cultivar, but LAB growth is inhibited in the Manzanilla cultivar due to its high content of phenolic compounds [3].

Several studies have demonstrated the important role of yeasts in the production of volatile compounds associated with good sensorial characteristics in natural-style spontaneously fermented olives [4–7]. Hence, we recently studied the use of selected yeast strains as starters for the production of naturally fermented Gordal, Manzanilla, and Hojiblanca green table olives [8]. It was found that the use of *Kluyveromyces lactis* L39 as a starter

resulted in a fermented product with a higher concentration of volatiles, mostly associated with pleasant aromas, compared to that from the corresponding uninoculated fermentation. However, seasonal variations were not considered. Season factors could affect the composition and microbiota of olives and, consequently, the volatilome of the fermented product. Furthermore, it is not clear whether yeast starters are essential to obtain a final product with better organoleptic characteristics than that obtained by spontaneous fermentation. Chytiry et al. [2] found higher levels of esters, alcohols, terpenes, and hydrocarbons, and better sensory characteristics including aroma and global assessment in Kalamàta and Conservolea black table olives inoculated with mixed cultures of yeasts (*Saccharomyces cerevisiae* or *Debaryomyces hansenii*) and LAB (*Leuconostoc mesenteroides* or *Lactobacillus plantarum*) compared to the spontaneously fermented olives. On the other hand, Hurtado et al. [9] did not find a significant preference for natural-style Arbequina green table olives inoculated with a single *Candida diddensiae* starter compared to the spontaneously fermented product.

Usually, to achieve an acceptable degree of bitterness that allows the product to be marketed, between 6 and 8 months of fermentation is necessary depending on the olive cultivar and the fermentation conditions. LAB or yeast starter cultures with the ability to reduce polyphenol content would be in potential demand in natural-style table olive processing, as they could significantly reduce the fermentation time. A significant bitterness-reducing effect has been found through the use of LAB starters, mainly *Lactobacillus plantarum* and *L. pentosus*, in natural-style table olives from different cultivars such as Ascolana tenera [10], Nocellara Etnea [11], Itrana [12,13], Bella di Cerignola [14], Leccino [12], Cypriot [15], and Picual [16]. However, the inoculation of Manzanilla olives with selected LAB with oleuropeinolytic activity was insufficient for reducing the high concentration of oleuropein in this cultivar [17]. Furthermore, the use of a β-glucosidase-positive yeast strain of *W. anomalus* in natural-style Manzanilla table olive processing did not have any bitterness-reducing effect [8].

Another aspect related to bitterness is the study of an instrumental method to estimate this attribute in natural-style table olives. A predictive model of the bitterness intensity of olives could have practical applications in the industry. This would allow one, quickly and objectively, to monitor the debittering process and find the ideal moment for packaging and marketing the olives without the need to evaluate them by a trained sensory panel. An easy and strong tool for bitterness assessment in virgin olive oil is based on total phenol content analysis [18,19]. However, to our knowledge, the relationship between total phenol content and bitterness intensity has not been investigated in table olives.

The objectives of the present study were as follows: (1) to verify the use of *K. lactis* L39 as a starter to enhance the volatile profile of natural-style Gordal and Manzanilla green table olives and to evaluate the sensory characteristics (color, firmness, and flavor) of the final product in comparison with the product obtained by spontaneous fermentation; (2) to evaluate other yeast starters selected based on different capacities for volatile production or distinctive traits such as β-glucosidase-positive activity; and (3) to evaluate the relationship between total phenol content and bitterness intensity in order to know whether the total phenol content could be used as an objective tool to estimate the bitterness intensity of natural-style green table olives from the Gordal and Manzanilla cultivars.

2. Materials and Methods

2.1. Selection of Yeast Strains

2.1.1. Growth and Volatile Profile of Yeasts in OCM

Twenty yeast strains isolated from fermenting olive brines collected during different harvest seasons in three table olive industries as well as in our experimental plant (Table S1) were analyzed for their growth and volatile profiles in a natural olive-derived culture medium (OCM). This OCM was obtained from pasteurized Manzanilla olives (2021–2022 harvest season), as detailed in a previous study [6], and stored at −30 °C until use. Before inoculation with the different yeast strains, salt was added to the defrosted

OCM to obtain 5% NaCl (*w*/*v*) in order to mimic the salt content usually encountered during the fermentation of natural-style table olives.

2.1.2. Enzymatic Activities of Yeasts

β-glucosidase, esterase, and lipase activities were assayed as described by Parafati et al. [20]. For this, 4-nitrophenyl-β-D-glucopyranoside (Indagoo Research Chemicals, Nanjing, China) was used as a substrate for the β-glucosidase activity test; 4-nitrophenyl acetate (Indagoo Research Chemicals) and 4-nitrophenyl butyrate (Fluorochem Ltd., Hadfield, UK) were used for the esterase activity; and 4-nitrophenyl palmitate (Thermo Fisher Scientific, Waltham, MA, USA) was used for the lipase activity.

2.2. Olive Processing

Green olives from the Gordal and Manzanilla cultivars were harvested in Arahal (Seville province, Spain) in late September 2022. The olives were selected to remove damaged fruits, washed, and placed in plastic vessels of 3.3 kg capacity filled with 2.1 L of 10% NaCl (*w*/*v*). Five different yeast starters were studied: (i) *K. lactis* L39 (treatments G-KL and M-KL, where G and M denote the Gordal and Manzanilla cultivars, respectively), (ii) *Kazachstania humilis* AG5 (G-KH and M-KH), (iii) *Nakazawaea molendinolei* IG9 (G-NM and M-NM), (iv) *Candida diddensiae* IG12 (G-CD and M-CD), and (v) *Candida adriatica* L30 (G-CA and M-CA). In each cultivar, spontaneous fermentations were carried out in parallel and served as controls (treatments G-Sp and M-Sp). All the fermentations were carried out in duplicate at ambient temperature (between 8 °C and 22 °C). The Gordal samples were kept in brine for a total period of 180 days, while the Manzanilla samples remained two months longer (~250 days) considering the higher polyphenol content (bitterness) of this cultivar. The yeast starters were inoculated twice, after 7 and 20 days of brining. For this, the selected yeast strains were grown in glucose yeast extract (GYE) broth containing 5% NaCl. After 72 h of incubation, the cultures were collected by centrifugation and the cell pellets were washed twice with saline, and finally resuspended in 10 mL saline. An aliquot (5 mL) of this suspension of the relevant yeast strain was inoculated in each vessel. Final concentrations ranged between 2×10^5 and 6×10^5 CFU/mL of each yeast strain in the brines. Brine sampling was performed during fermentation to monitor the main chemical characteristics (pH, titratable acidity, and salt) and microbial populations. At the end of fermentation, the volatile and phenolic compounds were determined in the brines and the phenolic compounds and sensory characteristics were analyzed in the fruits.

2.2.1. Physicochemical Characteristics

The physicochemical characteristics (pH, titratable acidity, and sodium chloride) of the olive brines were determined as described in [21].

2.2.2. Major Fermentation End-Products

Organic acids (lactic, acetic, citric, and succinic acids), ethanol and glycerol in the brines were quantified by HPLC using a Rezex ROA-Organic Acid column (300 mm × 7.8 mm i.d., Phenomenex, Torrance, CA, USA) and detection by a refractive index detector. The column was kept at 50 °C and 0.005 M H_2SO_4 was used as the mobile phase at 0.6 mL/min [3].

2.2.3. Volatile Compounds

The volatile compounds of the fermentations were analyzed by headspace solid-phase microextraction combined with gas chromatography–mass spectrometry (HS-SPME/GC-MS) [3]. The fiber used was a DVB/CWR/PDMS-80 µm (needle length 1 cm) (Agilent Technologies, Santa Clara, CA, USA). The SPME sampling conditions were as follows: 2 mL of brine and 0.4 g NaCl were transferred into a 15 mL glass vial and spiked with 20 µL of 6-chloro-2-hexanone (20 mg/L). The vial was closed and equilibrated for 5 min at 40 °C. The extraction and desorption times of the volatiles were 30 min and 5 min, respectively. The volatiles were separated using a VF-WAX MS capillary column (30 m, 0.25 mm i.d., 0.25 µm

film thickness; Agilent Technologies). The compounds were identified by comparing the mass spectra with those of the standard NIST 17 MS library and by the comparison of the retention indices with those of the NIST Standard Reference Database or authentic reference standards when available. Semi-quantitative determination was carried out by the internal standard method (IS, 6-chloro-2-hexanone). All the samples were analyzed in duplicate.

2.2.4. Phenolic Compounds

The total phenol content of the olive samples was determined as previously reported in Ruiz-Barba et al. [3]. Briefly, the polyphenols were extracted with methanol–water (60:40, *v*/*v*), and the hydroalcoholic extract was analyzed using the Folin–Ciocalteu procedure. The results were expressed as mg/kg of gallic acid equivalents of fresh weight sample. Single phenolic compounds in the OCM or brine were analyzed by HPLC using a Luna Phenyl-Hexyl column (5 µm, 250 mm × 4.6 mm, Phenomenex) and detection at 280 nm as described in the above-mentioned reference.

2.2.5. Determination of Instrumental Color and Firmness

The surface color of the olives was measured using a Color-View Model 9000 spectrophotometer (BYK-Gardner, Inc., Silver Spring, MD, USA) with a measurement area of 11 mm diameter, 45° circumferential illumination, and observation angle of 0°. All the measurements were performed on the CIE 1976 L*a*b* scale (L*: lightness [0 = black, 100 = white], a* [-a* = greenness, +a* = redness], and b* [-b* = blueness, +b* = yellowness]) using the illuminant type C and with a visual angle of 10º. Ten replicate measurements of each sample, each made on one olive, were taken. Hue angle (h°) was calculated as $\tan^{-1}(b^*/a^*)$. Chroma (C) was calculated as $(a^{*2} + b^{*2})^{1/2}$. Total color difference (ΔE) was calculated as $((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}$, and the color of uninoculated olives was used as a reference (G-Sp: L* = 53.1, a* = 7.0, b* = 41.2; M-Sp: L* = 43.8, a* = 6.7, b* = 23.6).

The firmness of the olives was measured using a Kramer shear compression cell coupled with TA.TXplus Texture Analyser (Stable Micro System, Surrey, UK).

The cross-head speed was 200 mm/min. The machine measures the shear compression force (expressed in newtons, N) to break 3 pitted olives. For each sample, the result corresponded to the mean of 10 measurements. Shear compression force was expressed as N/g of pitted olives.

2.2.6. Sensory Analysis

The quantitative descriptive analysis (QDA) approach was used to measure the intensity of the sensory characteristics of the olives. The panel was composed of 14 trained panelists (six women and eight men) belonging to the Instituto de la Grasa staff and with ample experience in the sensory analysis of table olives. The samples were served in cups coded with a 3-digit random number in individual booths under incandescent white lighting. The olives were presented in a balanced, randomized order to the panelists in order to avoid any order effect. Tap water was used for mouth rinsing between each sample evaluation. The sensory attributes that were evaluated included the following descriptors: odor, bitterness, saltiness, acidity, firmness, and overall acceptability. These attributes were assessed using a non-structured line scale from 1 (no perception) to 11 (extremely strong perception). The samples were analyzed in duplicate.

2.2.7. Evaluation of Olive Bitterness by Total Phenol Content Analysis

In order to build up the predictive model, 36 samples of naturally fermented Manzanilla and Gordal table olives obtained from different olive industries plus 8 samples of naturally fermented Manzanilla and Hojiblanca table olives from a previous study in our department [8] were analyzed for the total phenol content and perceived bitterness intensity through sensory analysis. To validate the predictive capacity of the model by com-

paring the predicted and measured bitterness intensities, the whole set of the samples of the natural-style Gordal and Manzanilla green olives studied in the present work was used.

2.2.8. Microbiological Analyses

In order to quantify LAB, *Enterobacteriacea*, and yeasts, the brines and their decimal dilutions (in sterile saline) were applied to agar plates containing the following media: Man, Rogosa, and Sharpe (MRS; Oxoid, Basingstoke, UK) agar supplemented with 0.02% (*w*/*v*) of sodium azide (AppliChem GmbH, Darmstadt, Germany) and 0.05% (*w*/*v*) of L-cysteine (Applichem) for LAB (incubated at 30 °C under anaerobic conditions for 72 h); Violet Red Bile Glucose (VRBG; Condalab, Torrejón de Ardoz, Spain) agar for *Enterobacteriaceae* (incubated aerobically at 37 °C for 24 h); and glucose yeast extract agar containing 0.01% (*w*/*v*) of oxytetracycline (OGYE; Applichem) for yeasts (incubated aerobically at 25 °C for 72 h). The population of each agar plate was then subjected to microbial count using a Scan 500 (Interscience, Saint Nom la Bretèche, France) colony counter.

2.3. Statistical Analyses

A principal component analysis (PCA) was performed in order to reveal any grouping of the yeast strains in OCM and to identify the main associations with volatile compounds. In addition, the PCA was used to explore the relationships between the variables (volatile compound concentrations) and the different treatments in the natural-style green table olives. These analyses were performed with the Unscrambler v. 11.0 (Camo Analytics, Oslo, Norway) or SIMCA 14.1 software (Umetrics, Umea, Sweden). The one-way analysis of variance (ANOVA, Tukey test) was applied to the microbiological, chemical, and sensory data in order to determine differences among treatments. Dunnett's test was used to compare the mean values of the chemical data from the inoculated samples against the uninoculated control sample. Significant differences were determined at the $p < 0.05$ level. The ANOVA was performed using the SPSS software v. 23.0 (IBM Corp., Armonk, NY, USA). In order to build up the bitterness predictive model, a regression analysis was applied using Excel 2019 for Windows.

3. Results and Discussion

3.1. Selection of Yeast Strains to Be Used as Starters

In the first stage, the selection of the yeast strains was based on their ability to grow in the OCM derived from pasteurized Manzanilla olives after 7 days of incubation at 25 °C. All yeast strains except *Zygoascus hellenicus* AG4 grew in this medium, so this strain was not used in further experiments. Then, PCA was used to classify the remaining yeast strains according to the volatile compounds produced and those strains closely associated with the desirable aroma attributes were selected. For this analysis, only the volatile compounds with an occurrence higher than two times in the entire data set were considered. With this criterion, 70 volatile compounds were considered, and their concentrations were transformed to Z-scores prior to the PCA. The results showed that the majority of the volatiles were found in the negative PC1 region (Figure S1). The strain *Kluyveromyces lactis* L39 was located in this region and clearly separated from the other yeast strains (Figure S1). Volatiles related to positive perceptions such as acetate esters (fruity and floral), isopentanol (whiskey and fruity), and isobutanol (ethereal and winey), among others, were associated with this strain. The association of this yeast strain with acetate esters was also found in our previous study [8], where OCM from a different olive season was used. The strain *Candida adriatica* L30 was located at the second quadrant showing no correlation at all with *K. lactis* L39, but the other volatiles with fruit perceptions such as the methyl and ethyl esters of C8-C10 fatty acids were related to this strain. In addition, this yeast strain was previously proposed as a solid candidate to be used as a starter culture to enhance the aromatic profile of naturally fermented green table olives [6]. The yeasts *Kazachstania humilis* AG5, *Kazachstania turicensis* AG6, and *Kazachstania bulderi* AG41 were grouped in the second quadrant indicating a close similarity between them, so we decided to select

just one of them (*K. humilis* AG5) for further experiments. This strain was associated with ethyl butanoate (fruity and sweet) and 1-butanol (sweet, balsamic, and whiskey).

Although the yeast strains located at the positive PC1 region or near zero did not show a clear association with volatile compounds, it is interesting to highlight that two strains, *Nakazawaea molendinolei* IG9 and *Candida diddensiae* IG12, showed a distinctive trait: both strains were able to grow in the OCM derived from unpasteurized Manzanilla olives, while the rest of strains did not grow in this medium. Besides, it is worth mentioning that, of the β-glucosidase-positive strains, *Nakazawaea molendinolei* IG9 showed the strongest β-glucosidase activity (Table S2). This biochemical activity is a desirable technological characteristic for yeast starters intended for table olives, as it has been related to the ability to hydrolyze oleuropein and diminish the bitterness of olives [22]. All the yeast strains presented esterase activity to a greater or lesser degree, but in no case was lipase activity observed (Table S2). Both esterase activity and lipase activity are desirable characteristics because they can increase the content of free fatty acids, which are precursors for the formation of several volatile compounds [23]. All in all, the strains *K. lactis* L39, *K. humilis* AG5, *N. molendinolei* IG9, *C. diddensiae* IG12, and *C. adriatica* L30 were selected to be used as starters for further experiments with natural-style green olive fermentations.

3.2. Natural-Style Green Olive Fermentations

3.2.1. Evolution of Microbial Counts and Physicochemical Parameters during Fermentations

The microbial dynamics of the main microbial groups (LAB and yeasts) detected in the brines of the Gordal and Manzanilla cultivars at three different times along the fermentations are shown in Table 1. In agreement with our previous studies with the Gordal and Manzanilla naturally fermented olives [3,7,8], LAB together with yeasts were detected in the Gordal olives, but only yeasts were detected in the Manzanilla samples. *Enterobacteriaceae* were only detected on day 7 in the Manzanilla brines but the counts were low (about 2 log CFU/mL) (data not shown). In the Gordal brines, the LAB population reached 10^6 CFU/mL after 30 days of fermentation without significant differences between the spontaneous and the inoculated fermentations, and this hardly changed thereafter, except in G-KH where the LAB population decreased by about 2 log cycles at the end of the process. In agreement with our previous study [8] but contrary to other studies [2,24,25], the yeast starters did not lead to an increase in LAB populations compared to spontaneous fermentation. Regarding yeast counts, all the starter-driven fermentations, except G-CD, presented higher yeast populations than the uninoculated treatment G-Sp (6.6–7.2 log CFU/mL versus 5.8 log CFU/mL) after 30 days, but no significant differences were found after 70 days and thereafter. The yeast populations reached 4.5–5.0 log CFU/mL at the end of the process. In the Manzanilla naturally fermented olives, the yeast populations were practically the same in all the brines after 30 days (5.4–5.8 log CFU/mL), remained constant up to 90 days, and slightly decreased at the end of the process.

In both cultivars, after 30 days of fermentation, the salt concentration in the brines reached 6.7–6.8% (*w*/*v*) NaCl, on average, and this value remained stable thereafter (data not shown). Data on physicochemical characteristics (pH and titratable acidity) showed clear differences between the two cultivars in agreement with the evolution of microbial groups (Figure 1). In the Gordal samples, pH quickly dropped to values below 3.9 units in all the samples in the first month of the fermentation, and gradually decreased with time reaching an average value of 3.52 units at the end of the process. Titratable acidity reached 0.84 g/100 mL, on average, after 180 days of the fermentation. No significant differences in the pH and acidity values of the inoculated samples with respect to the uninoculated sample G-Sp were found at the end of the fermentation. This result contrasts with that of Bonatsou and Panagou [26] who found that selected yeast starters belonging to *Candida boidinii* and *Saccharomyces cerevisiae* affected the final pH and titratable acidity of Kalamata natural black olives. The authors reported that only three out of the five yeast starters studied led to a final product with pH values within the limits of the standard trade for table olives (pH < 4.3) [27].

Table 1. Counts (log CFU/mL) of the main microbial groups in the brine samples of the uninoculated and inoculated natural-style Gordal and Manzanilla olive fermentations along the process [a].

Gordal olives						
	Yeasts			LAB		
Treatments	**30 days**	**60 days**	**180 days**	**30 days**	**60 days**	**180 days**
G-Sp	5.77 ± 0.14 a	5.95 ± 0.49	4.17 ± 0.77 ab	6.15 ± 0.14	5.93 ± 0.12	5.80 ± 0.66 bc
G-KL	6.59 ± 0.07 b	5.94 ± 0.30	4.47 ± 0.56 ab	6.29 ± 0.02	5.35 ± 0.52	6.21 ± 0.54 c
G-KH	7.08 ± 0.18 c	5.72 ± 0.18	5.05 ± 0.13 b	6.10 ± 0.10	5.77 ± 0.16	3.70 ± 0.55 a
G-NM	6.96 ± 0.16 bc	6.18 ± 0.45	4.88 ± 0.01 b	6.15 ± 0.08	5.84 ± 0.04	4.64 ± 0.48 ab
G-CD	6.09 ± 1.01 abc	6.26 ± 0.39	4.89 ± 0.37 b	6.27 ± 0.08	5.60 ± 0.25	5.57 ± 0.04 bc
G-CA	7.21 ± 0.31 bc	6.37 ± 0.40	3.68 ± 0.13 a	6.34 ± 0.08	5.70 ± 0.13	5.24 ± 0.65 abc
Manzanilla olives						
	Yeasts			**LAB**		
Treatments	**30 days**	**90 days**	**250 days**	**30 days**	**90 days**	**250 days**
M-Sp	5.45 ± 0.04 a	5.49 ± 0.01	4.83 ± 0.27 ab	ND	ND	ND
M-KL	5.44 ± 0.02 a	5.61 ± 0.26	4.36 ± 0.54 ab	ND	ND	ND
M-KH	5.47 ± 0.01 a	5.41 ± 0.05	5.03 ± 0.12 b	ND	ND	ND
M-NM	5.81 ± 0.05 b	5.29 ± 0.11	4.37 ± 0.28 ab	ND	ND	ND
M-CD	5.63 ± 0.20 ab	5.26 ± 0.21	4.56 ± 0.06 a	ND	ND	ND
M-CA	5.51 ± 0.11 ab	5.59 ± 0.11	4.50 ± 0.48 ab	ND	ND	ND

[a] data are means ± standard deviation. Different letters in the same column, for each olive cultivar, indicate significant differences ($p < 0.05$). ND = not detected.

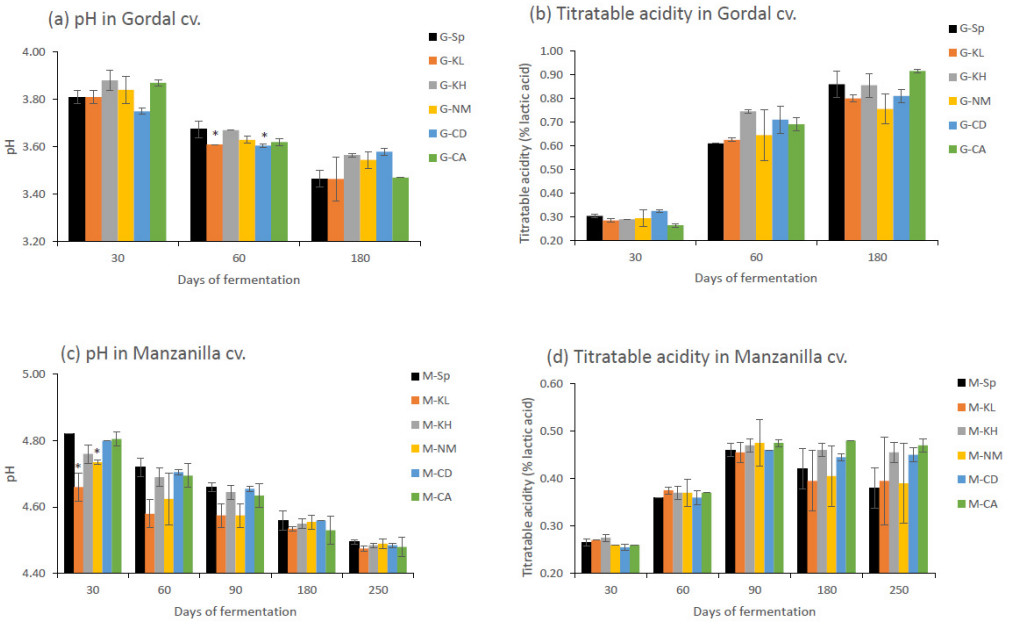

Figure 1. Changes in pH and titratable acidity in the brines of the uninoculated and inoculated samples of the natural-style Gordal (**a**,**b**) and Manzanilla (**c**,**d**) table olives during fermentation. The values are means of two replicate fermentations and error bars represent standard deviations. An asterisk above the bar denotes that the mean value is significantly different from the corresponding control sample (G-Sp or M-Sp) at the 0.05 level according to ANOVA (Dunnett´s *t*-test).

In the Manzanilla samples, the absence of LAB resulted in lower acidification during fermentation compared to Gordal. Thus, at the end of the process, the average values of titratable acidity and pH reached 0.42 g/100 mL and 4.49 units, respectively. This result agrees with our previous studies [3,7,8], and it implies that an acidification treatment is necessary in natural-style Manzanilla table olive processing in order to reach acceptable

pH values to ensure the stability of the final product. As in the Gordal cultivar, the addition of yeast starters in the Manzanilla samples had no significant influence on the evolution of these physicochemical parameters during fermentation in comparison with spontaneous fermentation (M-Sp).

3.2.2. Fermentation End-Products

As expected, due to the presence of LAB, lactic acid was the major fermentation end-product in the Gordal olives. At the end of the fermentation, the concentrations of this acid in the brines ranged between 0.71 and 0.93 g/100 mL (Figure S2). After lactic acid, ethanol (0.51–0.71 g/100 mL) and acetic acid (0.07–0.10 g/100 mL) were the most abundant end-products. Glycerol was also detected in trace amounts ($\leq$0.05 g/100 mL). In the case of the Manzanilla olives, ethanol was the most abundant end-product (0.66–0.87 g/100 mL) followed by glycerol (0.20–0.32 g/100 mL), citric acid (0.10–0.14 g/100 mL), acetic acid (0.05–0.07 g/100 mL), and succinic acid ($\leq$0.02 g/100 mL); no lactic acid was detected. In agreement with our previous study [8], in both cultivars, the effect of the yeast starters on the concentrations of the above-mentioned metabolites was not significant ($p > 0.05$).

3.2.3. Volatile Compounds

A total of 62 volatile compounds for the Gordal olives and 55 compounds for the Manzanilla olives were identified and semi-quantified at the end of the fermentations using the HS-SPME-GC-MS technique (Tables S3 and S4, respectively). The ANOVA revealed 37 and 29 volatiles for the Gordal and Manzanilla olives, respectively, which showed significant differences between the treatments ($p < 0.05$), and the PCA was applied using the contents of these volatile compounds as the variables. In the Gordal olives, the total variance of the two main PCs was 69.1% (Figure 2). The sample treated with *K. lactis* L39 (G-KL) was located in the positive part of PC1, clearly separated from the other samples, and was mainly associated with alcohols (benzyl alcohol, phenylethyl alcohol, (Z)-3-hexen-1-ol, 1-hexanol, and 1-octanol), terpenes (α-terpineol, isogeraniol, linalool, ionone, and linalool oxide), esters (phenethyl acetate), and phenols (creosol and 4-ethylphenol). All of these compounds presented significantly higher contents ($p < 0.05$) in G-KL than in the uninoculated sample G-Sp, as revealed by the ANOVA (Table S3). When the volatile profile of G-KL was compared with that of the fermentations inoculated with the same yeast strain under the same conditions (i.e., same cultivar, olive growing location, initial NaCl concentration, vessel material and size, and environment fermentation) from a previous olive season [8], clear differences were observed, with only four compounds in common (benzyl alcohol, phenylethyl alcohol, benzaldehyde, and 4-ethylphenol) showing significant increases (Table S5). It can be noted that, in the present study, the G-KL treatment resulted in a richer and more varied volatile profile than in the previous season. This discrepancy may be due to differences in environmental conditions between seasons (e.g., rainfall, temperature, radiation, and soil mineral concentration) that affect the composition of fresh olives and the microbial diversity and population dynamics during the fermentation process [28]. Seasonal variation in chemical compositions is a well-known phenomenon in plants, and it is associated with the biosynthesis, stability, and degradation of secondary metabolites in olives [29]. The remaining inoculated samples, which were located close to the origin, showed some similarity to the noninoculated ones and were hardly associated with the volatile compounds in the PCA biplot (Figure 2). Among the volatile compounds that significantly increased their concentration in each of these treatments compared to the noninoculated treatment, we can highlight 3-methylbutanal and 3-methyl-3-buten-1-ol in G-KH; decanal and 3-methyl-3-buten-1-ol in G-NM; prenol and geraniol in G-CD; and decanal and 3-methylbutanal in G-CA (Table S3).

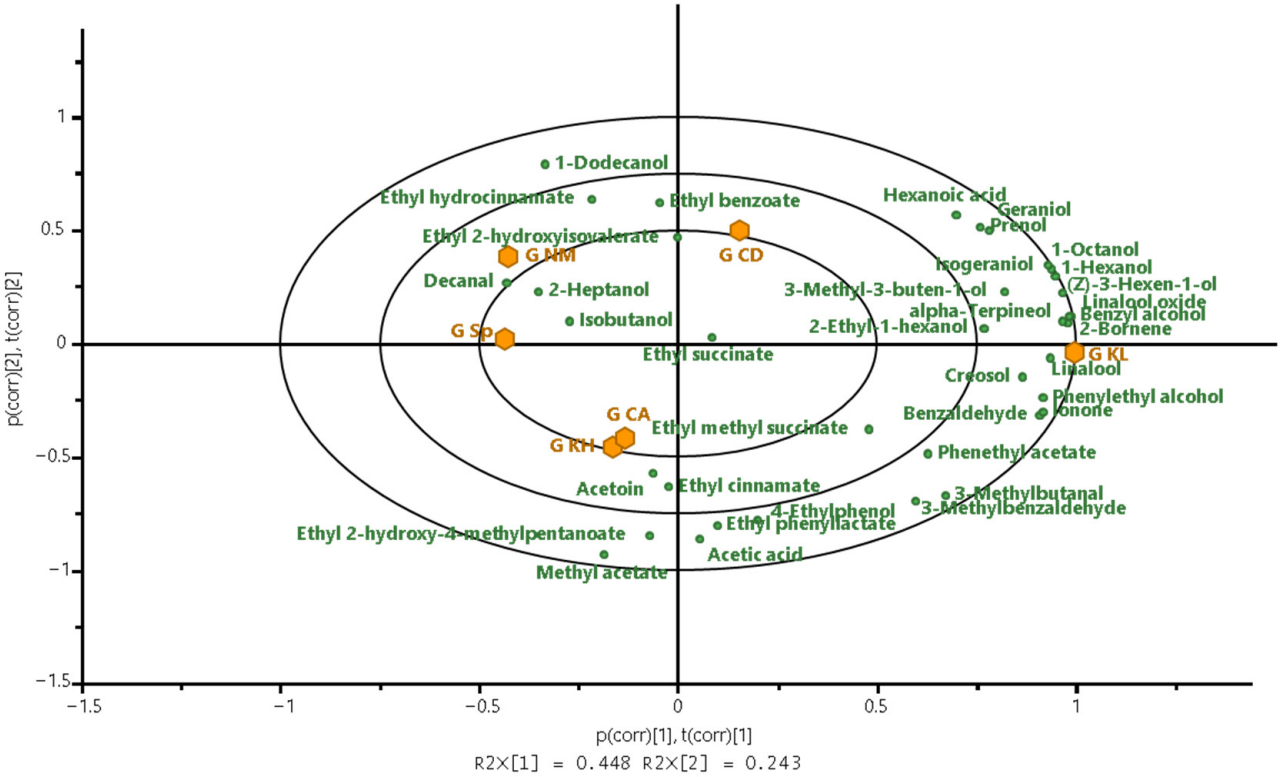

Figure 2. PCA biplot of the volatile compounds in the uninoculated and inoculated samples of the natural-style Gordal table olives at the end of the fermentation.

Regarding the Manzanilla olives, the PCA biplot, with a total variance of 63.3%, showed that the inoculated samples were clearly separated from the noninoculated one M-Sp (Figure 3), indicating a greater effect of the yeast starters on the volatile compounds in this olive cultivar compared to the Gordal cultivar. The sample M-KL was mainly associated with phenylethyl alcohol, ethyl octanoate, benzaldehyde, methyl 2,5-dimethyl-3-furoate, and ethyl 2,4-dimethyl-3-furoate, their contents being significantly higher than those of M-Sp (Table S4). It must be highlighted that the content of these last three compounds also increased significantly for the same treatment in a previous season [8] (Table S6). The sample M-CA was quite similar to M-KL, showing significantly higher concentrations of phenylethyl alcohol, methyl 2,5-dimethyl-3-furoate, ethyl 2,4-dimethyl-3-furoate, 2-methylbutanoic acid, and linalool oxide compared to the noninoculated sample. This result confirmed our previous finding in the sense that *C. adriatica* L30 could be a good candidate to enhance the aromatic profile of natural-style Manzanilla green table olives [6]. The samples M-KH and M-NM, which were very similar to each other, were mainly characterized by the higher contents of isobutanol and some acetate esters such as isobutyl acetate and isopentyl acetate, although the differences with respect to M-Sp were not significant in M-KH. Finally, the treatment M-CD was not related to any volatile compound, as corroborated by ANOVA.

Overall, in the Gordal cultivar, treatment with *K. lactis* L39 caused the greatest enrichment of volatile compounds compared to the noninoculated olives, which confirmed the results found in the previous season. In the Manzanilla cultivar, again *K. lactis* L39 produced the best volatile profile, although *C. adriatica* L30 gave a similar result.

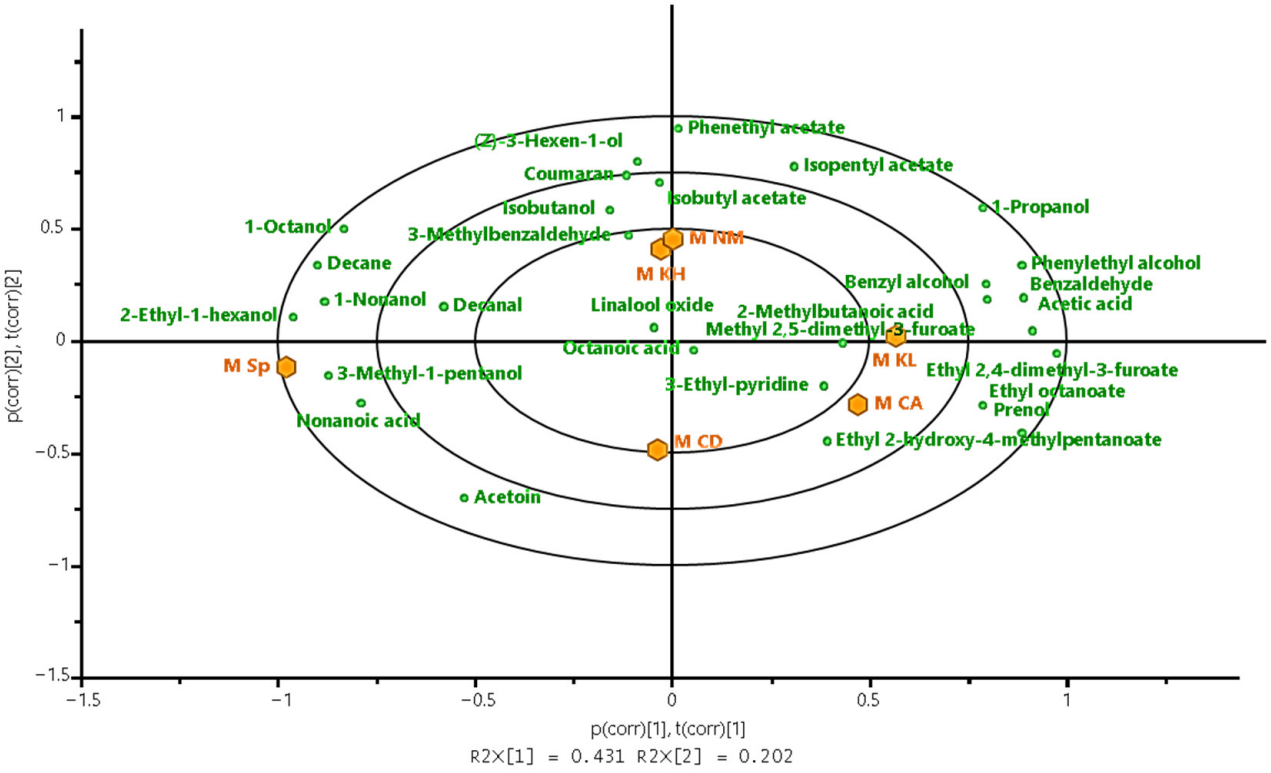

Figure 3. PCA biplot of the volatile compounds in the uninoculated and inoculated samples of the natural-style Manzanilla olives at the end of the fermentation.

3.2.4. Phenolic Compounds

As expected, the Manzanilla olives showed a higher total phenol content than the Gordal olives in both pulp and brine at the end of fermentation, but there was no significant influence of the yeast starters on total phenol content or on the concentration of individual phenols (oleuropein, hydroxytyrosol, tyrosol, or verbascoside) (Figures 4 and S3). This result agrees with our previous study [8], where no significant reduction in total phenols and oleuropein was found in the natural-style green table olives from Gordal, Manzanilla, and Hojiblanca cultivars treated with the single yeast starters of *K. lactis* L39, *P. kluyveri* L13, and *W. anomalus* L25. It must be stressed that three out of the five yeast starters used in the present study, namely *N. molendinolei* IG9, *C. diddensiae* IG12, and *C. adriatica* L30, and also *W. anomalus* L25, were positive for β-glucosidase activity using 4-nitrophenyl-β-D-glucopyranoside as a substrate. In contrast to our results, Ciafardini and Zullo [25] found that the total concentration of phenolic compounds in the brines of Taggiasca black table olives after 120 days of fermentation was lower in the fruits processed with the yeast starters *C. adriatica* 1985, *C. diddensiae* 2011, and *W. anomalus* 1960 than in the uninoculated olives. When our yeast strains were grown in OCM (prepared as mentioned in Section 2.1) for 7 days at 25 °C, no degradation of oleuropein was found compared to a control (Table 2). However, the hydroxytyrosol content was significantly increased in the OCM inoculated with *N. molendinolei* IG9 and *C. adriatica* L30. This can be attributed to the hydrolysis of hydroxytyrosol 4-glucoside, since a significant decrease in the peak area of this glucoside analyzed by HPLC, which eluted just before hydroxytyrosol, was observed (Table 2). These results suggest that natural media derived from olives such as OCM are more appropriate than the conventional synthetic media or phenolic analogs for testing the oleuropein-degrading ability of yeast cultures (or bacteria). In this sense, model brines containing several phenolic compounds (oleuropein, hydroxytyrosol, etc.) could also be used, as suggested by Anagnostopoulos and Tsaltas [30].

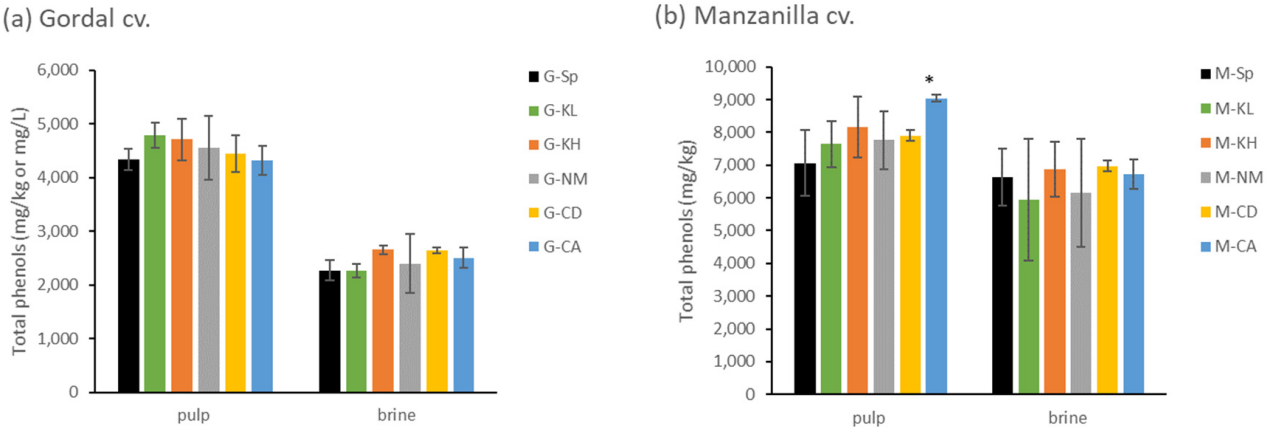

Figure 4. Content of total phenols in the pulp and brine of the uninoculated and inoculated samples of the natural-style Gordal (**a**) and Manzanilla (**b**) table olives at the end of the fermentation. The values are the means of two replicate fermentations and the error bars represent standard deviations. An asterisk above the bar denotes that the mean value is significantly different from the corresponding control sample (G-Sp or M-Sp) at the 0.05 level according to ANOVA (Dunnett´s *t*-test).

Table 2. Concentrations of single phenolic compounds in OCM derived from Manzanilla olives (2021–2022 harvest season) after fermentation for 7 days at 25 °C by selected yeast strains [a].

Sample	Oleuropein [b]	Hydroxytyrosol [b]	Tyrosol [b]	Verbascoside [b]	Hydroxytyrosol 4-Glucoside [c]
Uninoculated control	6677 ± 32	73 ± 1	26 ± 1	21 ± 1	8.68 ± 0.03
N. molendinolei IG9	6678 ± 61	117 ± 1 *	24 ± 1	14 ± 2	6.92 ± 0.00 *
C. diddensiae IG12	6372 ± 209	74 ± 1	24 ± 3	13 ± 3	8.70 ± 0.27
K. humilis AG5	6862 ± 233	71 ± 2	28 ± 2	25 ± 0	9.16 ± 0.37
C. adriatica L30	7052 ± 259	118 ± 5 *	33 ± 0 *	35 ± 20	7.88 ± 0.05 *
K. lactis L39	7012 ± 45	75 ± 0	20 ± 1 *	24 ± 0	9.46 ± 0.01 *

[a] Values are means ± standard deviation of duplicate fermentations. An asterisk (*) denotes that the mean value is significantly different from the control sample at the 0.05 level according to ANOVA (Dunnett´s *t*-test). [b] mg/L of OCM. [c] Peak areas ($\times 10^5$).

3.2.5. Sensory Characteristics of Final Products

The sensory characteristics including color parameters, instrumental firmness, and sensory attributes (odor, bitterness, saltiness, acidity, firmness, and overall acceptability) of the naturally fermented Gordal and Manzanilla olives were analyzed at the end of the fermentation. On average, the Gordal olives showed higher color parameters than the Manzanilla olives, with the exception of parameter a* (redness), which indicates that the Gordal olives presented higher lightness, yellowness, and chromaticity (Table 3). This indicates that these parameters are cultivar-dependent. However, both in the Gordal and Manzanilla olives, the individual color parameters did not show significant differences ($p > 0.05$) between the treatments. Color differences with respect to the noninoculated olives could be revealed by means of parameter ΔE*, this parameter being a combination of the parameters L*, a*, and b*. A ΔE* of two has been considered as a noticeable visual difference in a number of situations [31]. In the Gordal olives, the mean values of two were obtained in all the inoculated samples, except in G-NM, where a mean value of one was found. In the Manzanilla olives, the samples M-KL, M-NM, and M-CD showed ΔE* values ≥ 2, while the ΔE* values were about 1 in the remaining treatments.

Table 3. Color parameters and instrumental firmness of natural-style Gordal and Manzanilla green olives after natural-style fermentation [a].

Gordal cv.							
	G-Sp	**G-KL**	**G-KH**	**G-NM**	**G-CD**	**G-CA**	**Mean comparisons (ANOVA) [b]**
L*	53.1 ± 2.2	53.4 ± 1.3	54.5 ± 1.2	54.0 ± 0.4	51.8 ± 2.1	54.4 ± 0.8	NS
a*	7.0 ± 0.4	6.6 ± 0.2	6.9 ± 0.3	6.4 ± 0.4	7.1 ± 0.1	6.9 ± 0.8	NS
b*	41.2 ± 2.2	40.2 ± 1.9	42.4 ± 1.5	40.8 ± 0.7	39.5 ± 1.8	41.8 ± 0.5	NS
C*	41.8 ± 2.1	40.7 ± 1.9	42.9 ± 1.4	41.3 ± 0.8	40.1 ± 1.7	42.4 ± 0.7	NS
h°	80.4 ± 1.1	80.7 ± 0.2	80.7 ± 0.7	81.1 ± 0.4	79.7 ± 0.3	80.7 ± 1.0	NS
ΔE*	-	2 ± 1	2 ± 2	1 ± 1	2 ± 2	2 ± 0	NS
Instrumental firmness	39.7 ± 2.4	37.8 ± 2.6	42.3 ± 0.5	43.0 ± 3.3	43.6 ± 5.8	41.8 ± 2.4	NS
Manzanilla cv.							
	M-Sp	**M-KL**	**M-KH**	**M-NM**	**M-CD**	**M-CA**	**Mean comparisons (ANOVA)**
L*	43.8 ± 0.8	44.1 ± 1.3	44.3 ± 0.4	45.3 ± 0.8	43.0 ± 2.5	42.7 ± 0.6	NS
a*	6.7 ± 0.6	7.3 ± 0.4	7.2 ± 0.3	7.7 ± 0.2	6.9 ± 0.3	6.7 ± 0.4	NS
b*	23.6 ± 2.2	25.2 ± 1.9	24.0 ± 1.0	26.8 ± 0.5	25.0 ± 2.6	23.5 ± 0.7	NS
C*	24.6 ± 2.3	26.3 ± 2.0	25.0 ± 1.0	27.9 ± 0.5	26.0 ± 2.4	24.5 ± 0.6	NS
h°	74.2 ± 0.0	73.9 ± 0.3	73.4 ± 0.1	74.0 ± 0.0	74.5 ± 2.3	74.0 ± 1.3	NS
ΔE*	-	2 ± 2	1 ± 1	4 ± 1	3 ± 1	1 ± 1	NS
Instrumental firmness	61.0 ± 0.6	61.2 ± 2.4	61.3 ± 5.9	61.4 ± 0.2	59.0 ± 0.3	61.7 ± 0.5	NS

[a] data are means ± standard deviation. [b] NS, not significant ($p > 0.05$).

The instrumental firmness of the Manzanilla olives was significantly higher than that of the Gordal olives (1.5-fold higher on average) (Table 3), but no significant differences ($p > 0.05$) were found between treatments for each cultivar. This suggests that the used yeast starters had no or low pectinolytic activity. Golomb et al. [24] demonstrated that inoculation with pectinolytic yeast strains produced considerable softening of olives during natural-style green olive fermentations. In agreement with our results, Chytiri et al. [2] reported that firmness values for Kalamàta and Conservolea olives were not affected by the yeast starters of *S. cerevisiae* and *Debaryomyces hansenii*, respectively, which were added together with the LAB starters. On the other hand, Lanza et al. [13] found olive softening in Itrana naturally fermented olives inoculated with a mixed starter of *L. plantarum* and *Candida boidinii*, explaining this effect by the presence of *C. boidinii* enzymatic action on the fruit. It is worth mentioning that both the Gordal and Manzanilla olives showed values of instrumental firmness higher than those of Spanish-style olives (mean values of 40 N/g and 60 N/g versus 30–35 N/g, respectively) [32].

Regarding the sensory profiles of the final products, the panel did not find any significant difference between treatments (Figure 5). The overall acceptability scored by the panelists during the QDA sessions identified the Gordal olives, with lower scores for bitterness and higher scores for odor and acidity, as the preferred olive cultivar. Our results contrast with those of Chytiry et al. [2], who found higher odor intensities and higher global scores in inoculated (co-inoculum of yeast and LAB strain) olives. Apparently, the significant increases in the contents of various volatile compounds in some of the inoculated samples compared to the noninoculated ones (e.g., G-KL vs. G-Sp or M-KL vs. M-Sp, Tables S3 and S4, respectively) could not be detected by QDA. This could be due to the fact that QDA is not a discriminative test and may not be appropriate for the comparison of descriptors between two samples. Another explanation could be that the increased concentrations of volatiles were below their odor thresholds. Besides, it should be considered that for many aroma compounds (i.e., compounds with odor-activity values greater than 1), the perceived intensity is not proportional to the concentration, and there is a decelerating relationship as the concentration increases [33] (pp. 3–23). Therefore, it may be more difficult for panelists to notice changes in concentration at higher concentrations than at lower concentrations.

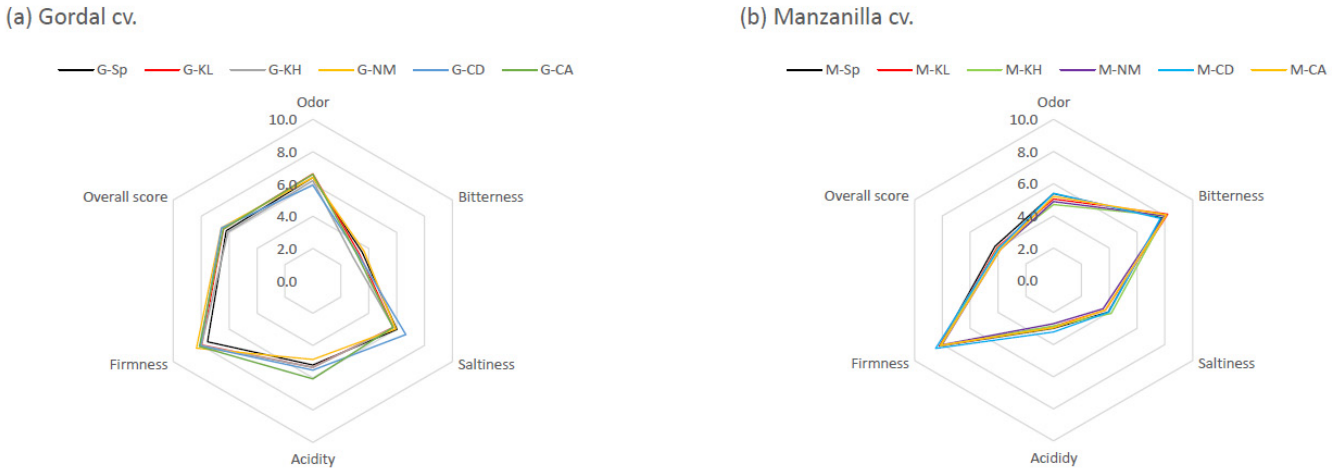

Figure 5. Sensory profile of the natural-style Gordal (**a**) and Manzanilla (**b**) table olives at the end of the fermentation.

Concerning olive bitterness, our results showed that the use of β-glucosidase-positive yeast strains such as *Candida adriatica* L30, *Nakazawaea molendinolei* IG9, and *Candida diddensiae* IG12 did not achieve any reduction in olive bitterness compared to that of spontaneously fermented olives (G-Sp or M-Sp). This agrees with our previous findings using another β-glucosidase-positive yeast strain (*Wickerhamomyces anomalus* L25) as a starter [8], but disagrees with the results reported by Ciafardini and Zullo [25], who found a lower bitterness intensity in Taggiasca black olives processed with the yeast starters *C. diddensiae* 2011, *C. adriatica* 1985, and *W. anomalus* 1960. All in all, these results appear to indicate that the effect of yeast starters on olive bitterness is strain-dependent, but the influence of the olive cultivar, the maturity stage of the olives, and seasonal factors should not be ruled out.

3.2.6. Evaluation of Olive Bitterness by Total Phenol Content Analysis

The relationship between the mean intensity of the perceived bitterness and total phenol contents of a total of 44 samples of natural-style Gordal and Manzanilla table olives is reported in Figure S4. A highly significant linear regression was obtained ($F = 185.6$; $p < 0.001$) showing a R^2 of 0.815. The following equation was obtained:

$$\text{Bitterness intensity} = 0.001\ TP - 0.871$$

where TP is the total phenol content (expressed as mg galic acid/kg olive pulp). This model was validated using the uninoculated and inoculated samples of natural-style Gordal and Manzanilla green olives studied in the present work (Figure 6). The results showed that in all the samples, the instrumental (predicted) bitterness intensity scores were not significantly different ($p > 0.05$) from the sensory mean scores. This indicates that the method used to predict the bitterness intensity of naturally fermented Gordal and Manzanilla olives, based on the total phenol content, was reliable. Therefore, the obtained prediction model could be used to successfully estimate the bitterness of natural-style Gordal and Manzanilla table olives without any sensory evaluation.

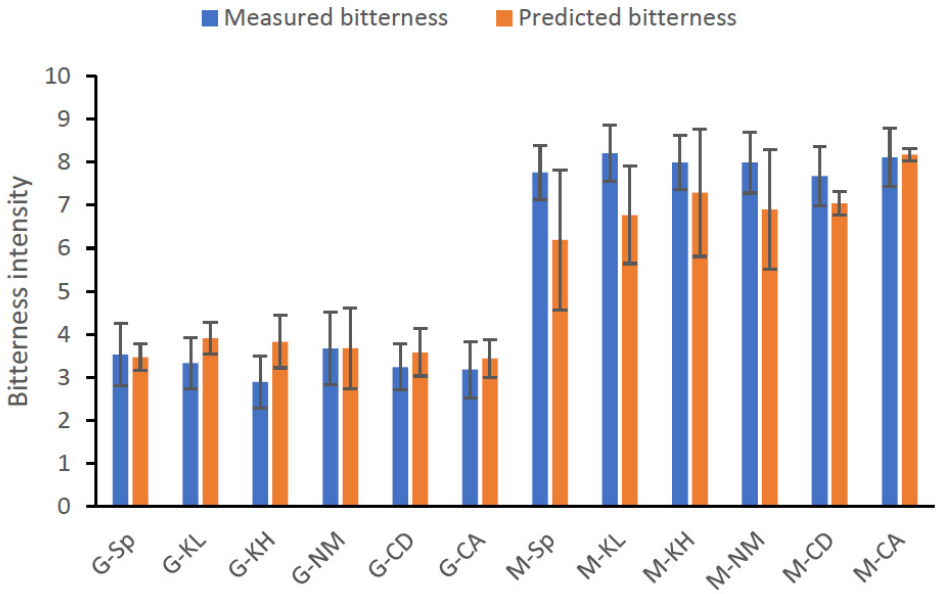

Figure 6. Comparison between the measured and predicted bitterness intensity in the uninoculated and inoculated samples of the natural-style Gordal and Manzanilla green table olives. Predicted bitterness intensity = 0.001 TP–0.8709, where TP is the total phenol content (mg galic acid/kg olive pulp). The values are means ± 95% confidence interval (measured bitterness, n = 30; predicted bitterness, n = 4).

4. Conclusions

In this work, we have studied the influence of different yeast starters on the fermentation of natural-style green table olives from the Gordal and Manzanilla cultivars, with special emphasis on the profile of volatiles, phenolic compounds, and organoleptic characteristics of the olives at the end of the process. Confirming the results obtained in a previous olive season, in the two cultivars, it was found that the use of yeast starters had a significant effect on the volatile contents of the fermented products. In particular, the strain *K. lactis* L39 produced the greatest enrichment of volatile compounds compared to the uninoculated product, in both the Gordal and Manzanilla cultivars. The strain *C. adriatica* L30 had a behavior similar to *K. lactis* L39 in the Manzanilla cultivar. In both cultivars, the use of β-glucosidase-positive strains such as *N. molendinolei* IG9, *C. diddensiae* IG12, and *C. adriatica* L30 did not significantly reduce the total phenolic content at the end of fermentation. Additionally, none of the yeast starters used in the present study had a significant influence on the organoleptic characteristics of fruits, including color parameters, texture, or sensory attributes perceived by a sensory panel through QDA. The total phenol content in the olives could be used to objectively estimate the bitterness intensity of the natural-style green table olives from the Gordal and Manzanilla cultivars. The challenge for future research will be to find a yeast species or strain with the ability to greatly degrade oleuropein to reduce bitterness and thus shorten the fermentation time, at least in the Manzanilla cultivar, since fermentation in this cultivar is only driven by yeasts. For this objective, the use of natural media derived from olives such as OCM is recommended to test the ability of yeast cultures to degrade oleuropein instead of phenolic analogs. However, additional research is needed to know whether changes in OCM composition due to seasonal or cultivar variations could affect the results.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10090439/s1, Figure S1. PCA biplot of volatile compounds produced by yeasts in OCM derived from pasteurized Manzanilla olives; Figure S2. Concentrations of organic acids, ethanol, and glycerol in the brines of the uninoculated and inoculated samples of the natural-style Gordal (a) and Manzanilla (b) table olives at the end of the fermentation. The values are means of two replicate fermentations and the error bars represent standard deviations; Figure S3.

Concentrations of single phenolic compounds in the brines of the uninoculated and inoculated samples of the natural-style Gordal (a) and Manzanilla (b) table olives at the end of the fermentation. The values are means of two replicate fermentations and error bars represent standard deviations; Figure S4. Relationship between total phenol content (mg galic acid/kg olive pulp) and perceived bitterness intensity in samples of naturally fermented olives from Gordal, Manzanilla, and Hojiblanca cultivars; Table S1. List of yeast strains isolated from fermenting brines of natural-style table olives tested for their potential to produce volatile compounds in a natural olive-derived culture medium (OCM); Table S2. β-Glucosidase, esterase, and lipase activities of the yeast strains investigated in the present study; Table S3. Volatile compounds in the brines of the uninoculated and inoculated samples of the natural-style Gordal green olives at the end of the process (180 days of fermentation); Table S4. Volatile compounds in the brines of the uninoculated and inoculated samples of the natural-style Manzanilla green olives at the end of the process (250 days of fermentation); Table S5. Volatile compounds that significantly increased their concentration in the naturally fermented Gordal table olives treated with the yeast starter *K. lactis* L39 (sample G-KL) compared to the noninoculated control (sample G-Sp) in two consecutive olive seasons; Table S6. Volatile compounds that significantly increased their concentration in the naturally fermented Manzanilla table olives treated with the yeast starter *K. lactis* L39 (sample M-KL) compared to the noninoculated control (sample M-Sp) in two consecutive olive seasons.

Author Contributions: Conceptualization, A.M. and J.L.R.-B.; formal analysis, A.H.S., J.L.R.-B., A.C.-D. and A.L.-L.; methodology, A.M. and J.L.R.-B.; investigation, A.M., J.L.R.-B., A.H.S. and A.L.-L.; resources, A.H.S. and A.L.-L.; data curation, A.M., J.L.R.-B., A.H.S., A.L.-L. and A.C.-D.; supervision, A.M.; writing—original draft, A.M.; writing—review and editing, A.M. and J.L.R.-B.; funding acquisition, A.M. All authors have read and agreed to the published version of the manuscript.

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