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Fermentation of Kalamata Natural Black Olives Using Selected Lactic Acid Bacteria as Starters

Despina Vougiouklaki [1], Sophia Letsiou [1], Iliana Mavrokefalidou [1], Efstathia Tsakali [1,2,*], Simen Akkermans [2], Jan F. M. Van Impe [2] and Dimitra Houhoula [1,*]

[1] Department of Food Science and Technology, Faculty of Food Sciences, University of West Attica, 12461 Athens, Greece; dvougiouklaki@uniwa.gr (D.V.); sletsiou@uniwa.gr (S.L.); fst18684087@uniwa.gr (I.M.)
[2] Department of Chemical Engineering, BioTeC + Chemical and Biochemical Process Technology and Control, KU Leuven, 9000 Gent, Belgium; simen.akkermans@kuleuven.be (S.A.); jan.vanimpe@kuleuven.be (J.F.M.V.I.)
* Correspondence: etsakali@uniwa.gr (E.T.); dhouhoula@uniwa.gr (D.H.)

Abstract: Fermented foods such as table olives are produced through a spontaneous process that has been improved over the years, ensuring the safety and quality of the final product. The aim of the present work was to study the action of starter cultures of lactic acid bacteria (*Lacticaseibacillus rhamnosus* GG ATCC53103, *Levilactobacillus brevis* ATCC8287, and *Lactiplantibacillus plantarum* ATCC14917) which were previously shown to have probiotic and antioxidant potential during the fermentation of natural Greek-style black olives (Kalamata) in brine containing 6% (w/v) NaCl at a temperature of 20 °C for a period of 150 days. At a molecular level, the main metabolites in every fermentation process were identified using an HPLC method. The results showed that the concentration of the metabolites increased gradually, developing a stable pattern after the 90th day of fermentation. In addition, the DL-p-hydroxyphenyllactic acid (OH-PLA) was identified as the phenolic acid with the highest concentration, independently of the selected starter culture. Microbial genomic DNA was also extracted from the olives' surface at the final stages of fermentation (150 days) and was subjected to 16S rRNA sequencing using the Nanopore MinION™ NGS tool, enabling a comprehensive analysis of the microbial community. According to the findings, the most abundant genera were *Lactobacillus* and *Leuconostoc*. To the best of our knowledge, this is the first study exploring these particular starters for olive fermentation.

Keywords: Kalamata olives; lactic acid bacteria; starter cultures; olive fermentation; Greek-style fermentation; microbiological analysis; NGS

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1. Introduction

Table olives, products of the olive tree (*Olea europaea* L.), are considered an important fermented product in the Mediterranean diet due to their high content of monounsaturated fats, primarily oleic acid, as well as phenolic compounds that are known to be a source of antioxidants for the human body [1]. Although the cultivation of olive trees has now been expanded in several parts of the world, the Mediterranean basin is still the main production area, with its share accounting for about 98% of the world's olive cultivation [2].

Greece is a major actor in the table olive industry as its annual production of 55,000 tons is the second highest in Europe, after Spain [3]. The sector has considerably developed during the last years, contributing significantly to the Greek national economy, as 85% of the production is exported. Kalamata table olives are mainly cultivated in the Peloponnese and Central Greece [3]. This particular type of table olive is characterized by its dark color and crunchy texture and holds a high export demand; it is assumed that Kalamata table olive production, primarily in the Greek prefectures of Etoloakarnania, Laconia, and Fthiotida, will exceed 100,000 tons within the coming 10 years [3,4].

The weight of the olives is approximately 3 to 6 g/fruit [5]. They are collected from the beginning of November until the beginning of winter, when they have formed the desired

deep black color but have not fully ripened. After undergoing natural fermentation, they are kept in brine where ripening is completed, resulting in their excellent organoleptic characteristics [6,7].

In general, fermented foods are developed through a spontaneous process that has been improved over the years, ensuring the safety and quality of the final product [8–10]. During this process, the most common microorganisms that are used are: *Enterobacteriaceae*, *Leuconostoc*, *Pseudomonadas*, *Pediocossus*, *Lactobacillus*, and fungi [11,12]. The Greek style fermentation process reduces or even eliminates the oleuropein content, which is the phenolic compound responsible for the bitter taste of olive drupes upon harvest. The removal of oleuropein also assists in producing a product with unique and high-quality organoleptic properties [12–15]. The fermentation process, which lasts 8-12 months, is dependent on the co-existence of lactic acid bacteria (LAB) and yeasts and their ability to produce acids that lower the pH [16,17] and produce antimicrobial compounds (e.g., bacteriocins) [18], both of which are related to the prevention of unwanted pathogens (e.g., *Listeria monocytogenes*, *Escherichia coli* O157:H7) [18,19]. It should be noted that the right inoculum of selected strains enables the improvement of the overall quality [20].

This study aimed to evaluate the effect of the use of three lactic acid bacteria, namely, *Lacticaseibacillus rhamnosus* GG ATCC53103, *Levilactobacillus brevis* ATCC8287, and *Lactiplantibacillus plantarum* ATCC14917, as starter cultures during the fermentation of Kalamata black olives in 6% initial salt brine according to Greek-style processing on the microbial and physicochemical quality, the sensory characteristics, the produced metabolites, and the microbiota of the final product. In a previous study [21] we characterized these lactic acid bacteria with probiotic and antioxidant potential. These lactic acid bacteria should have a double role as starter cultures with probiotic potential, which enables the control of the fermentation process, but also to deliver a final product with probiotic characteristics. In addition, these bacteria are expected to speed up the fermentation process compared to the traditionally used bacteria, which is a crucial factor for the industry to achieve effective and low-cost production processes. It is worth noting that this is the first time that these specific lactic acid bacteria have been used as starters cultures during the fermentation of Kalamata black olives, resulting in organoleptic characteristics in the final product which have not been reported before. Moreover, in order to assess the microbial community of these fermented Kalamata olives, next generation sequencing (NGS) was used for the identification of bacteria and the designation of origin based on 16S rRNA sequencing.

2. Materials and Methods

2.1. Bacterial Strain and Culture Conditions

For the growth of *L. rhamnosus* GG ATCC53103, *L. brevis* ATCC8287, and *L.plantarum* ATCC14917 (LAB), MRS broth (De Man Rogosa Sharp) was used. Before each experiment, incubation took place at 37 °C for 48 h under anaerobic conditions, while stock cultures were maintained in MRS broth containing 20% (*v*/*v*) sterile glycerol at −80 °C [21–23].

2.2. Bacterial Strain and Culture Conditions

Kalamata natural olives were collected based on the ripening stage (i.e., ¾ of the mesocarp had attained black color) in the first twenty days of November. The olives were transported to the laboratory in plastic containers with a capacity of 20 kg within 24 h of harvesting. Upon receipt, the olives were rinsed off with potable water to remove foreign matter and quality screening was performed to remove defective, damaged, and insect-infested fruit. Then, the olives were placed in 10 L fermentation vessels, each containing 5 kg of olives and about 3 L of brine (6% NaCl w/v), in order to apply the traditional Greek-style anaerobic processing.

To study the effect of lactic acid bacteria as starters on the natural fermentation of black olives, the following three cases were designed:

1. Natural fermentation with the olives' endogenous microflora (control)

2. Fermentation with the addition of *Lacticaseibacillus rhamnosus* GG ATCC53103 starter culture
3. Fermentation with the addition of *Levilactobacillus brevis* ATCC8287 starter culture
4. Fermentation with the addition of *Lactiplantibacillus plantarum* ATCC14917 starter culture

2.3. Microbiological Analysis

The microbiological analysis was performed according to the following procedure: For the brine sampling, 1 mL of brine was collected from each fermentation vessel and was aseptically transferred to 9 mL of sterile Ringer's solution. For the fruit sampling, 10 g of olive flesh was aseptically cut and homogenized in 90 mL of sterile Ringer's solution (Stomacher 400 circulator, Seward Ltd., Norwich, UK) for 90 s at room temperature. Thereafter, serial dilutions of 1:10 were performed, where 1 mL of sample was transferred to 9 mL of sterile Ringer's solution (0.9% g/L NaCl). Finally, decimal dilutions were made to inoculate a double series of plates for each microbiological substrate used to enumerate each microorganism. For the determination of LAB, a quantity of 1 mL from a series of two-fold dilutions of the homogenized sample was inoculated into de Man–Rogosa–Sharpe (MRS) selective agar, followed by incubation at a temperature of 30 °C for 72 h under anaerobic conditions. In order to prevent yeast growth, 0.05% w/v cycloheximide was added to the substrate after sterilization. Accordingly, for the determination of yeasts, 0.1 mL from the serial dilutions of the homogenized sample was inoculated using the surface coating technique on selective Rose–Bengal chloramphenicol agar (RBC agar), containing the antibiotic chloramphenicol to inhibit the growth of bacteria. This was followed by incubation at a temperature of 25 °C for 3 days and counting of the colonies. Finally, *Enterobacteriaceae* were placed on selective agar for a full day at 37 °C. Agar media that contained 30–300 colonies were used to enumerate microbial counts. The findings for olives and brine were log transformed and expressed as log CFU/g or mL for olives and brine, respectively.

2.4. pH and Salt Measurement

For pH and salt measurement, a digital meter (PC 80 + DHS Stirrer) was used. Specifically, the pH of brine samples was measured directly in the samples while the olives' pH was measured using a homogenous fluid slurry. The salt measurements were presented as a percentage (w/v) of NaCl.

2.5. Instrumentation and Analytical Conditions

2.5.1. Chromatographic Analysis (HPLC-UV/DAD)

The chromatographic analysis was performed on an HPLC system with a diode array detector (L-2455) (Hitachi LaChrom Elite, Tokyo, Japan). The HPLC-based separation was performed using a flow rate of 0.500 mL/min^{-1} on an SVEA C18 Gold column (150 × 4.6 mm 5 μm, Sweden). The temperature was set at 30 °C while a gradient mode was applied. The mobile phase consisted of water containing 0.1% formic acid (solvent A) and methanol containing 0.1% formic acid (solvent B) in a linear gradient flow as: 10% B for 0 min; 30% B for 10 min; 40% B for 20 min; 40% B for 25 min; 100% B for 40 min; 100% B for 45 min; 90% B for 50 min; 10% B for 55 min. The injection volume was 20 μL and elution was monitored at 280 nm [21–23]. Samples consisted of cell-free supernatants (CFSs) of the brine of each fermentation. The preparation included centrifugation at 13,000× *g* for 10 min followed by filtration of the supernatants (filter pore size 0.22 μm). The concentrations of the metabolites were calculated through standard curves. Different concentrations of standards (4-hydroxybenzoic acid, DL-p-hydroxyphenyllactic acid, phenyllactic acid, 3-(4-hydroxyphenyl) propionic acid, hydrocinnamic acid, methylcinnamic acid, salicylic acid, 1,2-dihydroxybenzene, 3,4-dihydrocinnamic acid, vanillic acid, 3,4-dihydroxyhydrocinnamic acid, ferulic acid, benzoic acid, and 4-hydrocinnamic acid) in water:methanol (90:10), ranging from 125–2000 ppm were used. The correlation coefficients (R^2) of all curves were above 0.99.

2.5.2. 16S rRNA Sequencing with Nanopore MinION™

NucleoSpin Food kit (Macherey-Nagel GmbH & Co. KG, Dueren, Germany) was used according to the manufacturer's instruction in order to extract the complete DNA from the olives. OD260/OD280 (Qubit™ 4 Fluorometer with the Qubit™ dsDNA BR Assay Kit) was calculated for the spectrophotometric evaluation of both the purity and quantity of the extracted DNA. The samples of the purified DNA were stored at −20 °C until further analysis.

For the amplification of the near full-length (V1–V9 region) of 16S rRNA, the MinION™ metagenomics kit (Oxford Nanopore Kits 16S Barcoding Kit) was used. From the 16S barcoding kit, 1 μL of each primer (10 μM) covering the full-length 16S gene (~1.5 kb) was used. PCR was performed using 10 μL of sample DNA (1 ng/μL) per reaction, 25 μL of Taq 2X master mix (Taq DNA Polymerase, QIAGEN), and 14 μL of nuclease-free water. Gene amplification was performed with an initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 30 s, extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. Amplicons were purified using 30 μL AMPure XP beads (NGS Clean-Up and Size Select, Macherey-Nagel, Duren, Germany), while quantification was achieved with a Qubit™ 4 Fluorometer with the Qubit™ dsDNA BR Assay Kit (Termo Fisher Scientifc, Waltham, MA, USA), which pooled all barcoded libraries with a total of 50–100 ng.

The R9.4.1 MinION™ system was used for 36 h (Oxford Nanopore Technologies, Oxford, UK) for sequencing while quick base-calling through MinKNOW software (v.18.01.6) was used for data collection. The generated data was analyzed in real time on the Nanopore EPI2ME cloud [4,24].

2.6. Statistics and Multivariate Analysis

Mean values ± standard deviation were used for the presentation of the continuous variables. For all the data, normality was assessed with the Kolmogorov–Smirnov test and graphically through Q–Q plots. Specifically, the sensory data for saltiness and crunchiness were normally distributed while bitterness, acidity, and hardness weren't normally distributed. To assess the comparison of sensory data among the different types of fermented olives, one-way analysis of variance (ANOVA) was applied after testing for the assumption of equality of variances (homoscedasticity) using Levene's test. For the data with skewed distribution, ANOVA was used on ranks (Kruskal–Wallis test). Multiple comparisons with Tukey's test were used to account for the increase in the likelihood of type I errors. In the case of the nonparametric test, multiple comparisons with Dunn's test were used. All reported *p*-values were based on two-sided tests and compared to a significance level of 5%. Prism 8 (GraphPad Software, Inc., San Diego, CA, USA) for macOS was used for statistical analysis.

2.7. Sensory Evaluation

The sensory evaluation pertained to the following factors: abnormal fermentation, saltiness, bitterness, acidity, hardness, fibrousness, and crispness. Ten trained panelists performed the evaluation of the table olives using the method of sensory analysis as established by the IOC [4]. The findings were based on scores on a 1–10 scale [4].

3. Results and Discussion

3.1. Microbial and Physicochemical Quality of Fermented Table Olives

In all samples where LAB starter cultures were added, the *Enterobacteriaceae* population decreased (<1 log CFU/g) after the 16th day of incubation in contrast to the control population, in which it decreased after 28 days of incubation. During the fermentation process in the fruit, LAB cultures were quite dominant, with an average population of 8.0 ± 0.5 log CFU/g in the beginning of fermentation and with a stable population of 7.0 log CFU/g by the end of fermentation. In the brine, the population change had an upward trend and finally stabilized at 6.0 ± 0.1 log CFU/mL. On the other hand, the

population density of the yeasts in the brine initially showed an increase and then steadily decreased to 4.0 ± 0.1 log CFU/mL by the end of the fermentation. Nevertheless, during fermentation supplemented with *L. brevis* or *L. rhamnosus*, the population density of the yeasts in the brine reached up to 7.0 ± 0.1 log CFU/mL or 6.0 ± 0.1 log CFU/mL, respectively, throughout the fermentation process. In addition, the population density of the yeasts in the fruit fluctuated at lower levels in all the different fermentations and did not appear to exceed 3.0 log CFU/g.

The final pH of all samples in the olive brine was less than 4.30, which complies with the physicochemical characteristics required for the safety of the final product [4]. More specifically, the pH value of the brine samples at the beginning of fermentation (5 days) was 5.33 for the control, 5.43 for *L. rhamnosus*, 5.40 for *L. brevis*, and 5.60 for *L. plantarum*. After 15 days, the pH decreased to acceptable limits for microbial safety (pH < 4.3).

3.2. Sensory Evaluation of Fermented Kalamata Table Olives

There were no off odors that indicate abnormal fermentation (i.e., butyric, putrid fermentation) or other defects detected by the panelists in any group of the fermented olives. The scores of the sensorial evaluations of fermented Kalamata table olives are represented in Figure 1. Specifically, the fermentations with the addition of *L. rhamnosus* and *L. brevis* showed higher scores for saltiness compared to the control ($p < 0.05$). In addition, the gustatory sensation evaluation showed that the *L. rhamnosus*- and *L. brevis*-fermented groups scored higher in bitterness than the *L. plantarum*-fermented group and the control group ($p < 0.05$, respectively). In the evaluation of the acidic taste, no significant differences were observed. However, the *L. rhamnosus*-fermented group scored higher in fibrousness evaluation according to the panelists compared to the control group ($p < 0.05$). Additionally, the *L. plantarum*-fermented group had higher scores in the kinesthetic sensations of hardness and crunchiness compared to the other fermented groups ($p < 0.05$).

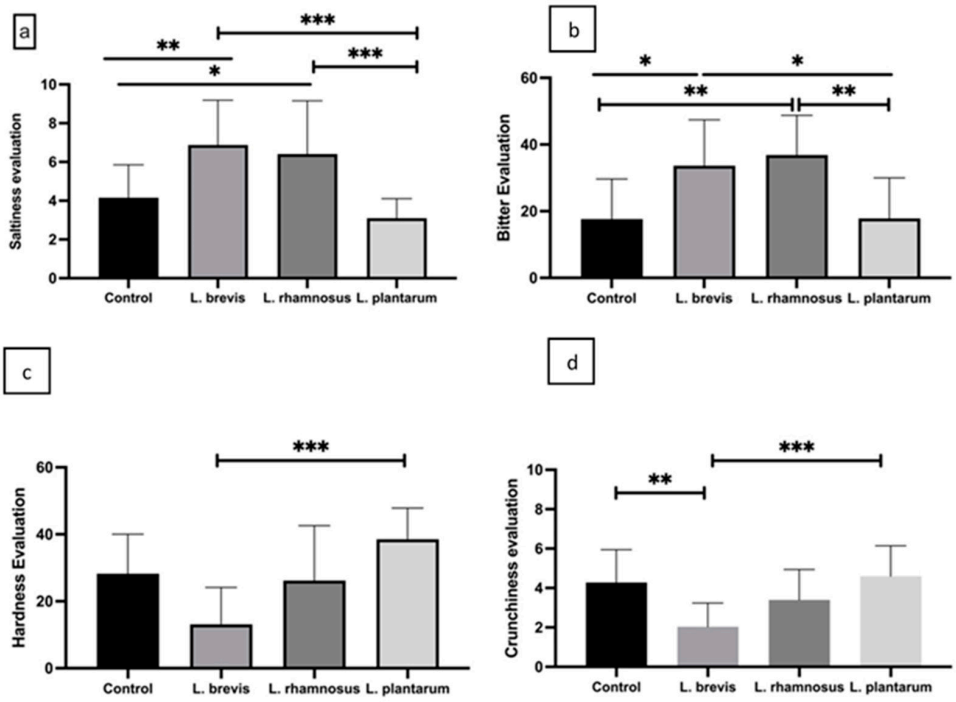

Figure 1. Sensory data expressed in bar graphs based on scores in an unstructured 1–10 scale. The experimental conditions (**a**–**d**) are: control, *L. brevis*, *L. rhamnosus*, *L. plantarum*. * $p < 0.05$ using ANOVA and after multiple comparison corrections using the Tukey test or Dunn's rule. ** $p < 0.01$ using ANOVA and after multiple comparison corrections using the Tukey test or Dunn's rule. *** $p < 0.001$ using ANOVA and after multiple comparison corrections using the Tukey test or Dunn's rule.

According to Figure 1, the sensory evaluation of saltiness, bitterness, hardness, and crunchiness showed statistically significant differentiation among the different types of fermented olives. However, the sensory descriptors of fibrousness as well as acidic taste did not show any statistical significance under the same conditions Specifically, the control group had statistically significant lower scores in the saltiness evaluation compared to *L. brevis*- ($p < 0.05$) and *L. rhamnosus*-fermented olives ($p < 0.01$). In addition, the *L. brevis*- ($p < 0.05$) and *L. rhamnosus*-fermented olives ($p < 0.01$) had statistically significantly higher scores in saltiness compared to *L. plantarum*-fermented olives ($p < 0.001$) (Figure 1a). In the case of bitterness evaluation, the control group had statistically significantly lower scores in bitterness compared to *L. brevis*- ($p < 0.05$) and *L. rhamnosus*-fermented olives ($p < 0.01$), while these fermented olives scored statistically significantly higher in bitterness compared to *L. plantarum*-fermented olives ($p < 0.001$ for *L. rhamnosus* and $p < 0.01$ for *L. brevis*-fermented olives) (Figure 1b). In the hardness evaluation, the scores of *L. brevis*-fermented olives were statistically significantly lower than *L. plantarum*-fermented olives ($p < 0.001$) (Figure 1c). Furthermore, in the case of the crunchiness evaluation, the control group had statistically significantly higher scores compared to *L. brevis*-fermented olives ($p < 0.01$), while *L. brevis*-fermented olives had statistically significantly lower scores compared to *L. plantarum*-fermented olives ($p < 0.001$) (Figure 1d). As expected, none of the sensory attributes of olives fermented with an *L. plantarum* starters were found to be significantly different from the control. This can be linked to the fact that *L. plantarum* is already the most abundant species in spontaneous fermentations.

3.3. Phenolic Content

Before the NGS analysis, the phenolic acid metabolites in the four experimental groups were determined: (1) natural fermentation with the olive's endogenous microflora (control group), (2) fermentation with the addition of *L. rhamnosus* GG ATCC53103 starter culture, (3) fermentation with the addition of *L. brevis* ATCC8287 starter culture, and (4) fermentation with the addition of *L. plantarum* ATCC14917 starter culture. The analysis was implemented during a 36-day period. Table 1 depicts the most dominant metabolites in each group while Figure 2 shows a representative chromatogram of the characteristic metabolites for each group, where even though the baseline drifts, a comparative quantification is still possible [22]. According to the findings, the concentration of the metabolites increased gradually towards an equilibrium after the 90th day of fermentation. Thus, identification of metabolites could be achieved from the first day of incubation of the microorganisms. In addition, DL-p-hydroxyphenyllactic acid (OH-PLA) was found at its highest concentration in all fermentation processes. The highest concentration (1194.7 ppm) was detected for *L. plantarum* and the lowest (426.8 ppm) was detected for *L. brevis* (Table 1). Moreover, a comparable quantity of vanillic acid (18.8–20 ppm) was identified for *L. brevis* and *L. plantarum* fermentations, while comparable quantities of benzoic acid (145.5–150.6 ppm) were identified for *L. brevis*, *L. plantarum,* and *L. rhamnosus* fermentation process (Table 1). Furthermore, similar quantities of 1,2-dihydroxybenzene (19.5–20.8 ppm) as well as ferulic acid (66.6–69.4 ppm) were found in all fermentations. On the other hand, methylcinnamic acid and salicylic acid were isolated in different concentrations in all fermentations, ranging from 182.7 to 350.4 ppm and 35.9 to 65.0 ppm, respectively. Unlike the other phenolic acids, the 4-hydrocinnamic acid was only detected during the fermentation process with *L. plantarum*.

As presented above, the results in Table 1 clearly demonstrate that the addition of the tested starter cultures considerably increased the concentrations of phenolic compounds, which have been associated with antioxidant activity. In particular, DL-p-hydroxyphenyllactic acid [25], methylcinnamic acid [26], salicylic acid [27], ferulic acid [28], and benzoic acid [29] increase in concentration and have been linked to antioxidant activity. This fact indicates the successful use of these starters as probiotics that lead to an increase in the phenolic content, and thereby to an increase in antioxidants.

Table 1. Concentrations (ppm) of metabolites that were detected in the natural fermentation of black olives for the four types of fermentation studied.

Name of Standard	Class	Control (ppm)	*Lactiplantibacillus plantarum* (ppm)	*Lacticaseibacillus rhamnosus* (ppm)	*Levilactobacillus brevis* (ppm)
(A) 4-Hydroxybenzoic acid	Phenolic acid	-	-	-	-
(B) DL-p-Hydroxyphenyllactic acid	Phenolic acid	573.5	1194.7	761.7	426.8
(C) Phenyllactic acid	Phenolic acid	-	-	-	-
(D) 3-(4-Hydroxyphenyl) propionic acid	Phenolic acid	-	-	-	-
(E) Hydrocinnamic acid	Phenolic acid	-	-	-	-
(F) Methylcinnamic acid	Phenolic acid	182.7	288.43	350.4	316.6
(G) Salicylic acid	Phenolic acid	35.9	46.3	65.0	58.31
(H) 1,2-Dihydroxybenzene	Benzenediols	20.2	19.6	19.5	20.8
(I) 3,4-Dihydrocinnamic acid	Phenolic acid	-	-	-	-
(J) Vanillic acid	Monohydroxy-benzoic acid	-	18.8	-	20.0
(K) 3,4-Dihydroxyhydrocinnamic acid	Phenolic acid	-	-	-	-
(L) Ferulic acid	Phenolic acid	27.2	68.7	69.4	66.6
(M) Benzoic acid	Phenolic acid	63.5	150.6	146.5	145.5
(O) 4-Hydrocinnamic acid	Phenolic acid	-	82.2	-	-

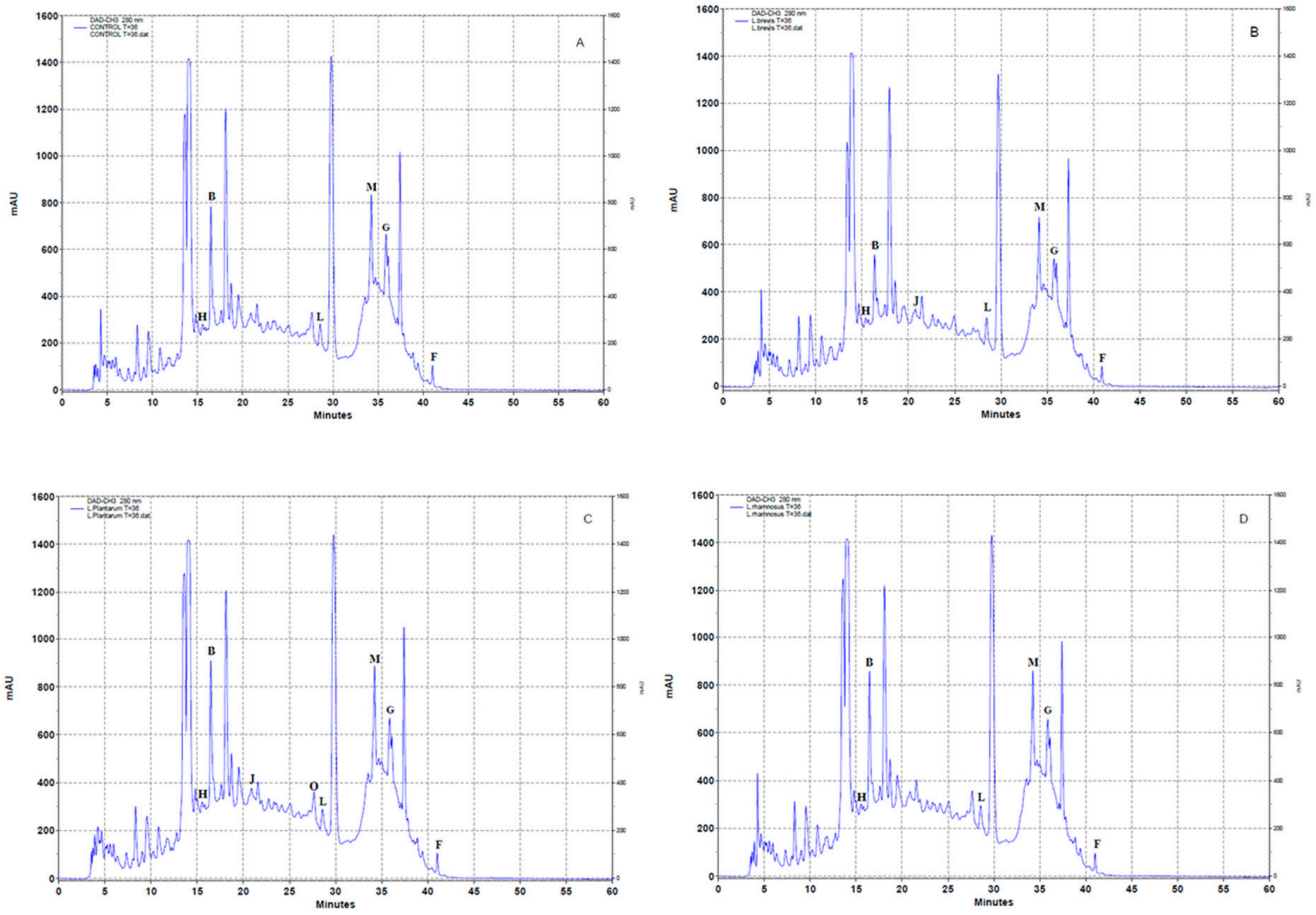

Figure 2. Chromatograms at 280 nm for the control (**A**), *L. brevis* (**B**), *L. plantarum* (**C**) and *L. rhamnosus* (**D**). High mass accuracy spectrum of the compounds (H) 1,2-dihydroxybenzene (15.7 min), (B) DL-p-Hydroxyphenyllactic acid (16.3 min), (L) ferulic acid (28.7 min), (M) benzoic acid (34.2 min), (G) dalicylic acid (36 min), (F) methylcinnamic acid (41.3 min), (J) vanillic acid (21.3 min), and (O) 4-hydrocinnamic acid (27.8 min).

3.4. Determination of Olive Microbiota Using 16S rRNA Sequencing

To assess the microbial community in the different fermentation conditions, 16S rRNA sequencing was conducted using a nanopore MinION™. Overall, our samples displayed concordant results between nanopore sequencing and conventional culturing. Specifically, in terms of genus, *Lactobacillus* spp., *Leuconostoc* spp., and *Enterobacter* spp. were detected in all samples. The most abundant genera, *Lactobacillus* and *Leuconostoc*, are commonly

identified in both fermented black and green olives using either classical microbiology or metagenomic analysis [24,30,31]. Moreover, this finding is in accordance with a recent work based on fermentation of the Kalamata variety [4]. Moreover, the presence of the genus *Enterobacter* spp. is quite well known and rather commonplace. However, it should be underlined that during classical microbiological analysis, the *Enterobacteriaceae* population was found to be lower than the detection limit of the method (<1 log CFU/g). Thus, the detection of the family *Enterobacteriaceae* in the samples potentially underlines the drawback of DNA-based metagenomic techniques, which is that the amplification of DNA can originate from dead or compromised cells [32]. According to the results, the most dominant species in all of our samples were *L. plantarum*, *Lactobacillus pentosus*, *Lactobacillus plajomi*, and *Lactobacillus paraplantarum* (Figure 3 and Tables 2–5).

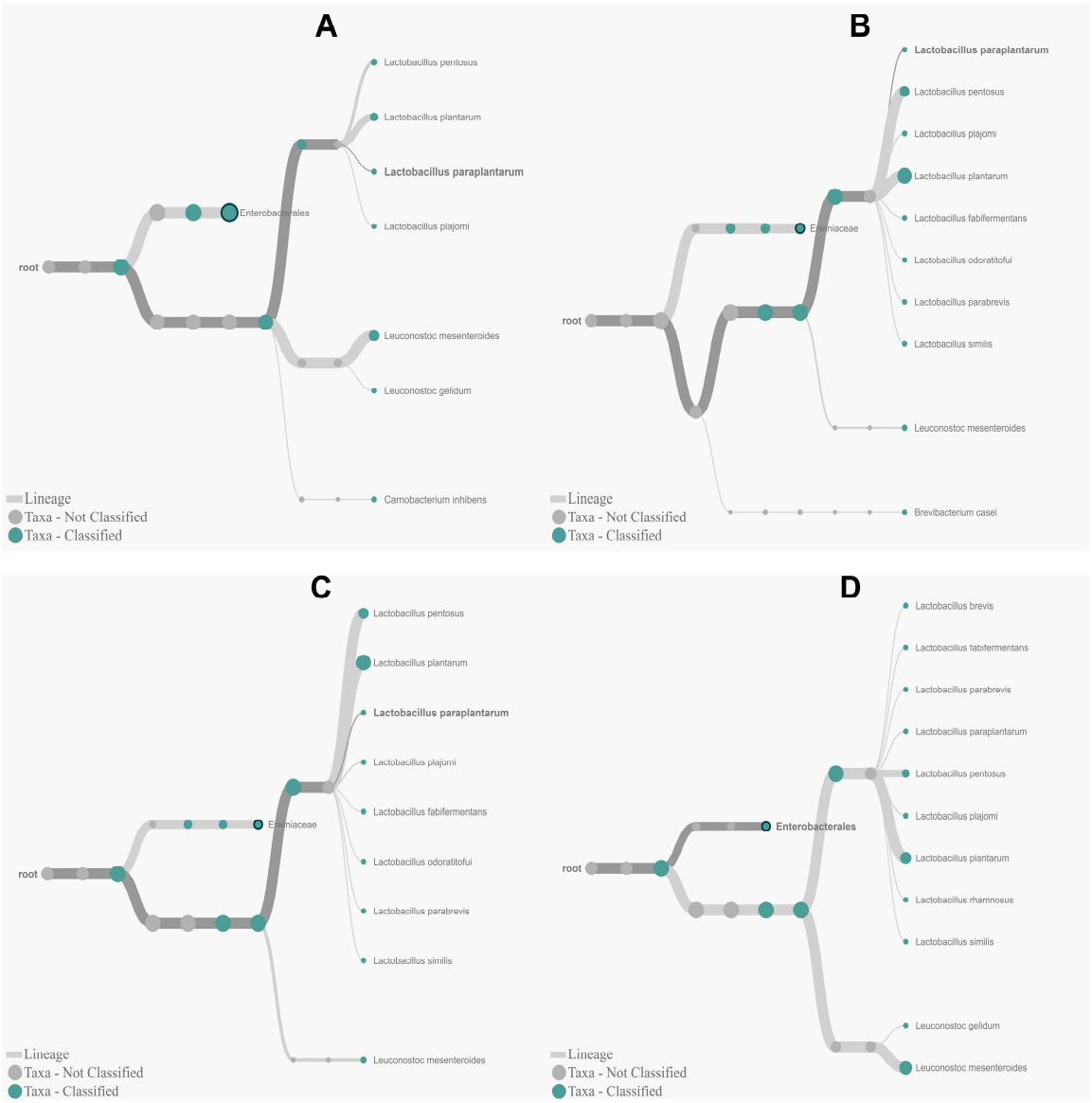

Figure 3. Most dominant species based on 16S rRNA sequencing with nanopore MinION™ in the four fermentation types: (**A**) natural fermentation with the olives' endogenous microflora (control); (**B**) fermentation with the addition of *L. plantarum* ATCC14917 starter culture; (**C**) fermentation with the addition of *L. rhamnosus* GG ATCC53103 starter culture; (**D**) fermentation with the addition of *L. brevis* ATCC8287 starter culture.

Table 2. The nanopore MinION sequence results from fermentation with the addition of the starter culture *Levilactobacillus brevis* ATCC8287.

Taxon	MinION Reads Total
Lactiplantibacillus plantarum	42,451
Lactiplantibacillus pentosus	18,122
Lactiplantibacillus plajomi	921
Lactiplantibacillus paraplantarum	817
Lacticaseibacillus rhamnosus	559
Lactiplantibacillus fabifermentans	284
Levilactobacillus brevis	190
Levilactobacillus parabrevis	147
Latilactobacillus graminis	105
Secundilactobacillus odoratitofui DSM 19909 = JCM 15043	99
Fructilactobacillus fructivorans	8
Lactiplantibacillus modestisalitolerans	72
Lacticaseibacillus paracasei	71
Paucilactobacillus suebicus	71
Secundilactobacillus paracollinoides	69
Companilactobacillus tucceti	64
Lactiplantibacillus argentoratensis	57
Lentilactobacillus rapi	54
Levilactobacillus hammesii	51

Table 3. The nanopore MinION sequence results from fermentation with the addition of the starter culture *Lactiplantibacillus plantarum* ATCC14917.

Taxon	MinION Reads Total
Lactiplantibacillus plantarum	45,886
Lactiplantibacillus pentosus	23,335
Lactiplantibacillus plajomi	878
Lactiplantibacillus paraplantarum	593
Lactiplantibacillus fabifermentans	283
Levilactobacillus parabrevis	178
Levilactobacillus fructivorans	96
Lactobacillus plantarum subsp. Argentoratensis	93
Levilactobacillus koreensis JCM 16448	92
Secundilactobacillus paracollinoides	91
Paucilactobacillus suebicus	87
Companilactobacillus versmoldensis	67
Lactiplantibacillus modestisalitolerans	60
Latilactobacillus graminis	57
Lentilactobacillus rapi	49
Companilactobacillus tucceti	48
Paucilactobacillus vaccinostercus	39
Secundilactobacillus mixtipabuli	37

According to a recent report on LAB in table olives, the species *L. plantarum*, *L. pentosus*, and *L. brevis* were the most abundant while the species *L. coryniformis*, *L. paraplantarum*, and *Lc. mesenteroides* were in lower quantities [11]. However, in the fermentation group with the addition of *L. brevis* (Table 2), the species *L. rhamnosus* and *L. brevis* were detected as well. The same applies to the fermentation group with the addition of *L. rhamnosus*, achieving, however, much lower reads for these species (Table 4). The species *L. rhamnosus* and *L. brevis* have been identified before in green olives of Algeria, Italy, and Spain, as well as in natural black and green olives of Algeria, Italy, Turkey, and Morocco, respectively [33–38]. It is worth noting that the *Lactobacillus* spp. dominate in a number of table olive processing methods. However, this apparent dominance may also be the consequence of a more extensive focus in the study of these species, leaving many other species undiscovered. The increased accessibility of more accurate identification techniques (mostly molecular

genotyping) as well as the existence of widely accessible and densely populated genomic sequence databases, however, may be the reason why there have been several studies of new species in recent years. Although in-depth examination of isolated microbes might be possible with the use of next-generation sequencing and meta-analysis techniques such as metagenomics and meta-transcriptomics, this research topic has not yet been thoroughly investigated.

Table 4. The nanopore MinION sequence results from fermentation with the addition of the starter culture *Lacticaseibacillus rhamnosus* GG ATCC53103.

Taxon	MinION Reads Total
Lactiplantibacillus plantarum	68,410
Lactiplantibacillus pentosus	34,893
Lactiplantibacillus plajomi	1,291
Lactiplantibacillus paraplantarum	908
Lactiplantibacillus fabifermentans	385
Levilactobacillus parabrevis	292
Lactobacillus plantarum subsp. *Argentoratensis*	155
Levilactobacillus fructivorans	134
Secundilactobacillus paracollinoides	131
Paucilactobacillus suebicus	131
Levilactobacillus koreensis JCM 16448	130
Lactiplantibacillus modestisalitolerans	86
Latilactobacillus graminis	83
Companilactobacillus versmoldensis	73
Lentilactobacillus rapi	68
Companilactobacillus tucceti	58
Companilactobacillus musae	52
Companilactobacillus furfuricola	51

Table 5. The nanopore MinION sequence results from natural fermentation with the olives' endogenous microflora (control).

Taxon	MinION Reads Total
Lactiplantibacillus plantarum	45,218
Lactiplantibacillus pentosus	16,695
Lactiplantibacillus plajomi	744
Lactiplantibacillus paraplantarum	693
Lactiplantibacillus fabifermentans	294
Levilactobacillus parabrevis	141
Lactobacillus plantarum subsp. *argentoratensis*	111
Latilactobacillus graminis	94
Levilactobacillus koreensis JCM 16448	85
Paucilactobacillus suebicus	85
Fructilactobacillus fructivorans	74
Secundilactobacillus paracollinoides	72
Lentilactobacillus rapi	61
Lactiplantibacillus modestisalitolerans	56
Paucilactobacillus vaccinostercus	54
Companilactobacillus tucceti	45
Companilactobacillus versmoldensis	35
Lactiplantibacillus xiangfangensis	35

4. Conclusions

Table olives hold significant economic value globally, particularly in Mediterranean countries. In the fermentation of natural black olives, LAB and yeasts compete for dominance. Despite the economic importance of table olives, fermentation technology is empirical and can often lead to fluctuations in organoleptic and physicochemical profile of the final product. To improve the fermentation process and produce a stable, high-quality

product, process control is essential. The use of *Lacticaseibacillus rhamnosus* GG ATCC53103, *Levilactobacillus brevis* ATCC8287, and *Lactiplantibacillus plantarum* ATCC14917 as starter cultures for the fermentation of Kalamata table olives, which have known probiotic potential, was explored and was found appropriate for producing fermented products with acceptable sensory characteristics. The addition of the selected starter cultures led to an increase in all measured phenolic compounds, which are associated with antioxidant activity. Specifically, DL-p-hydroxyphenyllactic acid (OH-PLA) was identified at the highest concentration in all the different fermentation processes. Moreover, according to the outcomes of the nanopore MinION™ NGS tool, the most abundant genera identified in the fermented Kalamata table olives were *Lactobacillus* and *Leuconostoc*. To the best of our knowledge, this is the first study exploring these particular starters with probiotic potential for the fermentation of olives. However, further studies are needed for confirmation and for the development of a final probiotic product.

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