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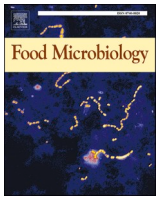

Microbial community and volatilome changes in brines along the spontaneous fermentation of Spanish-style and natural-style green table olives (Manzanilla cultivar)

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ABSTRACT

Microbial community and volatilome of brines were monitored during the spontaneous fermentations of Spanish-style and Natural-style green table olives from Manzanilla cultivar. Fermentation of olives in the Spanish style was carried out by lactic acid bacteria (LAB) and yeasts, whereas halophilic Gram-negative bacteria and archaea, along with yeasts, drove the fermentation in the Natural style. Clear differences between the two olive fermentations regarding physicochemical and biochemical features were found. *Lactobacillus, Pichia,* and *Saccharomyces* were the dominant microbial communities in the Spanish style, whereas *Allidiomarina, Halomonas, Saccharomyces, Pichia,* and *Nakazawaea* predominated in the Natural style. Numerous qualitative and quantitative differences in individual volatiles between both fermentations were found. The final products mainly differed in total amounts of volatile acids and carbonyl compounds. In addition, in each olive style, strong positive correlations were found between the dominant microbial communities and various volatile compounds, some of them previously reported as aroma-active compounds in table olives. The findings from this study provide a better understanding of each fermentation process and may help the development of controlled fermentations using starter cultures of bacteria and/or yeasts for the production of high-quality green table olives from Manzanilla cultivar.

1. Introduction

Among vegetables, table olives are the most widespread fermented food in Western countries, particularly in the Mediterranean area (Campus et al., 2018). According to data from the Association of Exporters and Industrialists of Table Olives (ASEMESA), the average production of table olives in Spain in the last five seasons was 561,100 tons, which represents 19.7% of world production of this product (ASEMESA, 2022).

At the global level, two different styles (or preparations) of fermented table olives stand out, namely, Spanish-style green olives and Natural-style (Sánchez et al., 2021). The Spanish-style processing includes an alkaline treatment with a solution of NaOH (or "lye") in order to eliminate the bitterness of the fruits followed by a stage of washing with water and, finally, placing in brine where a typical spontaneous fermentation takes place. The Natural-style olives, also known as "table olives in brine" or "Greek-style table olives", are elaborated by placing directly olives (e.g. green, turning-color, or black fruits) into brine, without any debittering pre-treatment. As a result, a long holding period in brine (5–8 months) is necessary in order to reduce bitterness to acceptable levels. Both processing technologies have been extensively studied in separate by many research groups but, to the best of our knowledge, a detailed comparison between the two types of olive fermentation under the same pre-harvest conditions (e.g. olive cultivar, growing location, olive maturity, damage, pesticide residues) and post-harvest variables (temperature, fermentation vessel, water, NaCl) has not been carried out to date.

The microbiota associated with both fermentation processes is quite complex and involves the growth of a great diversity of bacteria, mainly LAB, and yeast species, which determine the final characteristics such as flavor, texture and safety (Bonatsou et al., 2017). LAB growth is strongly influenced by the physico-chemical conditions (i.e. salt content, pH,

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aerobic/anaerobic conditions, temperature) and olive cultivar (Portilha-Cunha et al., 2020). Despite extensive research into the microbial ecology of these olive styles, the roles of the different microbial groups and species in contributing to the fermentation process and final quality are not fully understood.

Research related to volatilome in both Spanish-style and Natural-style green olives has been relatively abundant in the last years (Spanish-style green olives: Sánchez et al., 2017, 2018; Martorana et al., 2017; López-López et al., 2018; De Castro et al., 2019; Garrido-Fernández et al., 2021; Natural-style green olives: Bleve et al., 2014, 2015; Randazzo et al., 2014, 2017; De Angelis et al., 2015; Martorana et al., 2015; Penland et al., 2020, 2022; Mikrou et al., 2021), yet few of these investigations included correlation studies between microbiota and volatilome.

Manzanilla cultivar is the most important olive cultivar devoted to table olive production in Spain. This cultivar is characterized by relatively high levels of polyphenols (mainly oleuropein, the major bitter compound in olives) which inhibit the LAB growth. Hence, Natural-style fermentation using Manzanilla cultivar is mainly driven by yeasts (Medina et al., 2010; Aponte et al., 2010; Alves et al., 2012; Montaño et al., 2021).

The objectives of this work were to study the microbial community and volatilome in the brines during the spontaneous fermentation of Manzanilla olives processed according to the Spanish-style and Natural-style procedures, which were run in parallel, and also to investigate the correlations between microbial communities and volatile compounds in each case. In addition, the fermentation biochemistry (i.e. the sugars consumption and formation of major end-products) was studied in order to get a better understanding of each fermentation process. The results of the present study may help the development of controlled fermentations using starter cultures of bacteria and/or yeasts for the production of high-quality green table olives from Manzanilla cultivar.

2. Materials and methods

2.1. Olive processing

The olives (Manzanilla cultivar) were harvested in Arahal (Seville province, Spain) at their mature-green stage and transported to our laboratories to be processed. Olives were subjected to quality control to remove damaged fruits and then placed into six cylindrical vessels made of polyethylene (21 kg fruits plus 14 L liquid capacity), of which three (coded S1–S3) were used for triplicate fermentations of Spanish-style olives and the other three (coded N1–N3) for triplicate fermentations of Natural-style olives. The olives in vessels S1–S3 were alkali treated with 1.8% (w/v) NaOH for 6 h. Then, the fruits were washed twice with tap water (washings duration: 75 min each) and, finally, a brine with 13% NaCl (w/v) as initial concentration was used to sustain the fermentation. The olives in vessels N1–N3 were directly immersed in brine containing 7.1% (w/v) of NaCl. Fermentations took place at ambient temperature, which ranged 8–22 °C.

2.2. Sampling

Brine samples from each vessel were taken during fermentation to control the main physicochemical and microbiological characteristics. Analyses of volatile compounds and total DNA extraction from the fermenting brines were carried out at three different times: at the beginning (7 days after brining in S1–S3, 15 days in N1–N3), at the middle stage (30 days in S1–S3, 60 days in N1–N3) and at the final stage (90 days in S1–S3, 208 days in N1–N3).

2.3. Physicochemical analyses

The physicochemical characteristics (pH, titratable acidity, combined acidity, salt concentration) of the olive brines were determined by the routine methods used in our laboratories (Sánchez et al., 2000).

2.4. Analysis of sugars, organic acids, ethanol and glycerol

Major sugars in brines (glucose, fructose, mannitol, sucrose) were determined by HPLC using a Rezex RCM column (Phenomenex, Torrance, CA) at 80 °C, deionized water as the mobile phase and a refractive index detector (Casado and Montaño, 2008). Lactic acid, acetic acid, citric acid, succinic acid, ethanol and glycerol were quantified by HPLC with an Aminex HPX-87H (Bio Rad Labs, Hercules, CA) column, 0.005 M H_2SO_4 as the mobile phase and a refractive index detector (Sánchez et al., 2000). All samples were analyzed in duplicate.

2.5. Analysis of volatilome

The volatilome profiles were determined by headspace solid-phase microextraction combined with gas chromatography-mass spectrometry (HS-SPME/GC-MS). For the analysis, 3 mL of brine was placed into a 15 mL glass vial with 50 μL of internal standard (5-nonanol, 2 mg/L) and volatiles were extracted, identified and quantified according to methods previously described (Montaño et al., 2021). The volatile compounds were semiquantified by comparison of peak areas to that of internal standard. Each sample was analyzed in duplicate.

2.6. Microbiological analyses of olive brines

Populations of the main groups of microorganisms were determined by plating the brines, and their decimal dilutions (in 0.9% NaCl), on the appropriate solid media with a spiral plater (Don Whitley Sci. Ltd., Shipley, England). The culture media used were de Man, Rogosa, Sharpe agar (MRS, Biokar diagnostics, Beauvais, France) with and without 0.02% sodium azide (Sigma-Aldrich) for LAB, oxytetracycline-glucose-yeast extract (OGYE, Oxoid Ltd., Basingstoke, England) agar for yeasts, and VRBG agar (Biokar) for *Enterobacteriaceae*. MRS plates were incubated under anaerobic conditions using airtight containers with the AnaeroGen gas generation system (Oxoid), while OGYE and VRBG plates were incubated aerobically. MRS and VRBG plates were incubated at 32 °C for up to 5 days, while OGYE plates were incubated at 25 °C for up to 5 days. The resulting numbers of colony forming units were counted with a Scan 500 (Interscience, St Nom la Bretèche, France) colony counter.

2.7. Metagenomic analysis

Total DNA from samples of fermenting olive brines was extracted and purified using the DNeasy PowerFood Microbial Kit (Qiagen). For the metagenomic analyses, purified DNA samples were processed at the Sequencing and Bioinformatic Service of FISABIO (Valencia, Spain) where the 16 S rRNA gene, for bacteria, the Intergenic Transcribed Spacers (ITSs), for fungi, Metagenomic Sequencing Library Preparation Illumina protocol and subsequent MiSeq sequencing was followed as previously described (Medina et al., 2018). Distribution of reads along the quality check protocol and DADA2 routine is shown in Table S1.

2.8. Statistical analyses

The one-way analysis of variance (ANOVA) was applied to compare the means of individual concentrations of volatiles or chemical classes during fermentation. Significant differences were determined at the $p < 0.05$ level. Principal component analysis (PCA) was performed using the contents of the volatile compounds presenting significant differences during fermentation as the variables in order to reveal any grouping of the samples during fermentation as well as to identify the main compounds associated with each group. Spearman's correlation coefficient was obtained as a measure of the correlation between microbiota and volatile compounds. Only microbial OTUs with abundances >1% in at

least 2 samples were considered. ANOVA and Spearman's correlations were performed using SPSS software v. 23.0 (IBM Corp., Armonk, NY, USA). Correlation heatmap diagrams, with hierarchical clustering using Ward's minimum variance linkage and Euclidean distance method, were performed with PermutMatrix software v. 1.9.3 (Caraux and Pinloche, 2005). PCA was performed with the Unscrambler software (version 11.0; Camo Analytics, Bedford, MA). The interaction network was generated by Cytoscape software v. 3.9.1 (Shannon, 2003) with nodes representing volatile compounds and microbial taxa, and edges representing correlation coefficients.

3. Results and discussion

3.1. Evolution of microbiological counts and physicochemical characteristics during fermentation

LAB growth in the Spanish-style olives provoked a fast acidification of brine with a concomitant decrease of pH from 5.96 to 4.18 after 30 days of fermentation (Fig. 1a, Table S2). Meanwhile, no LAB growth occurred in Natural-style green olives, which resulted in lower acidification of brine (pH decreased from 4.90 to 4.46). In Natural-style olives, a significant increase in titratable acidity (from 0.19 to 0.38% expressed as lactic acid) was found between 20 and 30 days of fermentation. This can be attributable to the formation of organic acids and CO_2 by yeast metabolism as discussed later. *Enterobacteriaceae* (ranging between <1.30 and 2.25 log CFU/mL) were only detected in Natural-style olives after 7 days of fermentation and were not detected in any other sample afterwards (data not shown). Yeasts were present throughout the fermentation in both processing styles. Although the initial yeast counts in Spanish-style samples were lower than those of Natural-style olives (2.81 versus 5.09 log CFU/mL), from the 30th day to the end of fermentation, yeast population showed similar counts in both cases reaching maximum values of 5.70–5.89 log CFU/mL (Table S2). It is worth to mention that the final pH in Natural-style olives was slightly over the pH required by the current normative (namely, pH < 4.3 according to the International Olive Council; IOC, 2004). Therefore, in order to improve the product safety by reaching pH < 4.3, the addition of lactic acid into the initial brine or into the fermenting brine at the beginning of the fermentation would be an advisable treatment in Natural-style green olives from Manzanilla cultivar.

3.2. Changes in fermentation substrates and major end-products

Fermentation substrates present in the fruits diffused into the brines and were subsequently metabolized (Fig. 1b, Table S3). However, differences between the two processing styles were found in the metabolism of mannitol and citric acid. Mannitol was partially metabolized at the final stage of fermentation in Spanish-style olives, but it remained stable in Natural-style olives in accordance with previous studies (Montaño et al., 2021). Citric acid was totally degraded in Spanish-style olives, but this acid increased its concentration from day 7–30 and then remained stable until the end of process in Natural-style olives. An increase in citric acid was also found by Penland et al. (2020) during natural fermentation from Tanche olive cultivar. Lactic acid was the major end-product in Spanish-style olives, whereas ethanol was the predominant end-product in the Natural style. However, the relatively high concentration of ethanol formed in Natural-style olives cannot be explained by this mechanism only. Acid or enzymatic hydrolysis of oleuropein and other glycosides during fermentation can produce glucose (Charoenprasert and Mitchell, 2012), which could be metabolized by yeasts with formation of ethanol. It is also worth mentioning that, although CO_2 was not analyzed in the present study, an amount of CO_2 similar to that of ethanol should be produced through the fermentation of sugars (Maicas, 2020). This CO_2 production, along with the

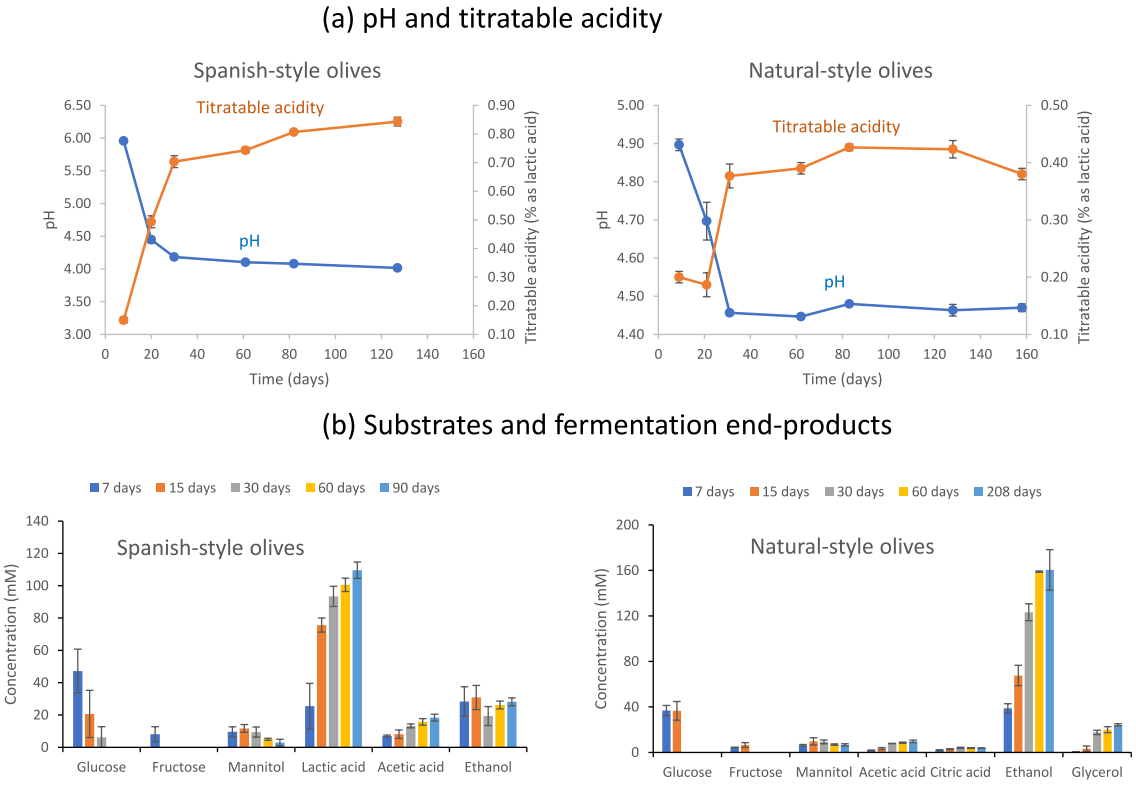


Fig. 1. Physicochemical and biochemical changes during fermentation of Spanish-style and Natural-style green table olives of the Manzanilla cultivar. (a) pH and titratable acidity in brine, (b) fermentation substrates and major end-products in brine. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

increases in the concentrations of citric, acetic and succinic acids in Natural-style olives, could explain the increase in titratable acidity from day 20–30 mentioned in the previous section.

3.3. Microbial communities during fermentation

Metataxonomy analysis of the fermenting brines was carried out at three different times along fermentation, as mentioned in section 2.2. The first sampling point was considered as the first stage of fermentation once NaCl has reached equilibrium between olive juice and brine. The second sampling point corresponded to a stage where the concentrations of the major fermentation end-products stabilized, and the last sampling point corresponded to the final stage of process. The relative abundance (%) of bacteria, archaea and fungi for Spanish-style and Natural-style green table olives are shown in Fig. 2. Only microbial OTUs with abundances >1% in at least 2 samples were considered. The sequencing data has been deposited in the ENA public repository under the project identification number PRJEB61009. A comprehensive record of all OTUs obtained by the metataxonomy analyses are shown in Tables S4–S7. The number of reads, number of OTUs, Good's coverage and different diversity indexes are shown in Tables S8 and S9 for the Spanish-style and the Natural-style samples, respectively.

In the Spanish style, a great variability was observed in the relative abundance of bacteria and fungi among replicate vessels at the initial and middle stages of the fermentation (Fig. 2). At the initial stage, halophilic and alkaliphilic Gram-positive bacteria represented the major group. Subsequently, the genus *Lactobacillus* gradually increased and it was dominant at the end of fermentation in all the three vessels. It has been extensively described that it is actually the species *L. pentosus* the one that largely dominates this fermentation in the geographical area of this study (Lucena-Padrós et al., 2014; Lucena-Padrós and Ruiz-Barba, 2019). *Lactobacillus buchneri* was found at relatively high level (25%) at the final stage of fermentation in sample S2. This could affect the volatile profile of this sample in comparison with S1 and S3, as revealed by the PCA of volatile compounds discussed later. Different fungi species were found with abundances highly variable between replicate vessels at the initial and middle stages of fermentation. *Pichia manshurica* was dominant in all the three vessels at the end of the fermentation, along with *Saccharomyces*.

Unlike Spanish-style processed olives, the microbial communities in Natural-style olives showed relative abundances quite similar between replicate vessels throughout the fermentation (Fig. 2). Among the Prokaryotes, only genera of Gram-negative bacteria and archaea were found. Most of the identified bacteria and archaea genera have been described in the past as halophiles or, at least, halotolerant, according to data shown at the HaloDom (v. 1.3) web page (http://halodom.bio.auth.gr/?view=home). This fact indicated a more than probable origin in the marine salt used to elaborate the initial brine. The presence of relatively high abundance of halophilic/halotolerant bacteria such as *Halomonas, Salinicola, Marinobacter, Aliidiomarina* and *Pseudomonas* in brines from Natural-style green olives of different varieties has been also reported by several authors (Cocolin et al., 2013; Medina et al., 2016; Randazzo et al., 2017). At the end of fermentation, *Allidiomarina*, *Halomonas* and *Marinobacter* were dominant in the brines. The yeast *Saccharomyces* was by far the dominant genus during the initial and middle stages of fermentation, while the genus *Nakazawaea* and the species *P. manshurica*

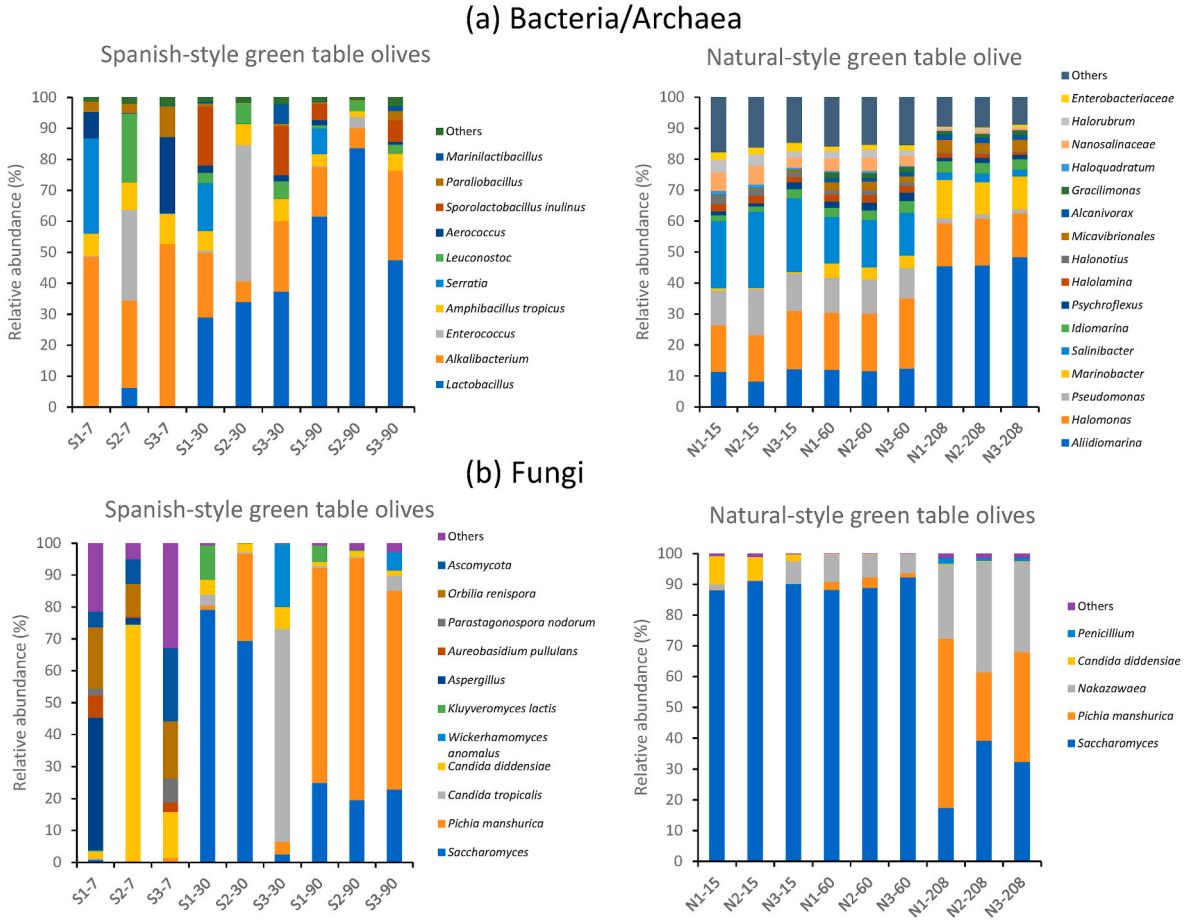


Fig. 2. Relative abundance (%) of bacteria and fungi during fermentation of Spanish-style and Natural-style green table olives of the Manzanilla cultivar. (a) Bacteria, (b) Fungi. Only microbial OTUs with abundances >1% in at least 2 samples were considered. The numbers behind sample code represent fermentation times (days). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

increased to a great extent, being dominant along with *Sacharomyces* at the end of fermentation.

3.4. Changes in volatile compounds during fermentation

A total of 93 volatile compounds were identified in the present study (Table S10). They were grouped into different chemical classes, namely, alcohols, esters, acids, carbonyl compounds, sulfur compounds, terpenes, and phenols, whose evolutions during fermentation in both processing styles are presented in Table 1. At the final stage of fermentation, the two olive types significantly differed in volatile acids and carbonyl compounds. Concentrations of individual volatile compounds for each processing type are shown in Table S11. Alcohols constituted the predominant chemical class in both processing styles. This chemical class increased gradually its concentration during fermentation in both cases. At the end, ethanol, (*Z*)-3-hexen-1-ol and isopentanol largely prevailed over all the identified alcohols in Spanish-style olives, whereas ethanol, isopentanol and isobutanol were the predominant alcohols in Natural-style olives. Phenylethyl alcohol was also formed in relatively high levels in both types of olives.

Esters significantly increased during fermentation in both cases. The predominant ester was ethyl acetate. After ethyl acetate, ethyl lactate was the most abundant ester in Spanish-style olives, which is consistent with the high concentrations of lactic acid formed in this type of table olives, but it was not detected in Natural-style olives. On the contrary, methyl 2,5-dimethyl-3-furoate and ethyl hexanoate were relatively abundant and only formed in Natural-style olives.

Acids significantly increased during fermentation in the Spanish style, but remained unchanged in the Natural style. Acetic acid was the main volatile acid in both types of olives. 2-Methylbutanoic acid was formed in relatively high amounts in Spanish-style olives, but was undetected in Natural olives. On the contrary, 3-methylbutanoic acid was only detected in Natural-style olives. It is worth to mention that the concentrations of hexanoic, octanoic, and decanoic acids significantly decreased between 60 and 208 days in Natural-style olives.

Carbonyl compounds did not significantly change in the Spanish style, but a notable increase was found in the Natural style at the final stage of fermentation. This was mainly due to the formation of the methyl ketones 2-pentanone, 2-heptanone and 2-nonanone, presumably as a consequence of oxidation of free fatty acids by *Penicilium* species growing on the surface of brine. The above-mentioned decrease in the concentrations of hexanoic, octanoic, and decanoic acids supports the assumption that the methyl ketones were produced by this mechanism.

Table 1
Evolution of chemical classes of volatile compounds during fermentation of Spanish-style and Natural-style green olives of the Manzanilla cultivar.[a].

	Spanish-style olives			Natural-style olives		
Chemical class	7 days	30 days	90 days	15 days	60 days	208 days
Alcohols	219 ± 57 a	283 ± 84 ab	375 ± 94 b	248 ± 58 a	401 ± 42 b	429 ± 31 b
Esters	32 ± 6 a	76 ± 10 b	132 ± 22 c	64 ± 13 a	111 ± 11 b	137 ± 15 c
Acids	7 ± 8 a	78 ± 10 b	145 ± 22 c	24 ± 11 a	29 ± 2 a	27 ± 2 a
Carbonyl compounds	9 ± 3 a	11 ± 2 a	8 ± 2 a	1.4 ± 0.3 b	0.07 ± 0.01 a	48 ± 16 c
Sulfur compounds	24 ± 8 a	21 ± 6 a	28 ± 11 a	13 ± 3 a	20 ± 2 b	13 ± 2 a
Terpenes	2 ± 2 a	3 ± 1 a	4 ± 2 a	0.9 ± 0.3 a	2.3 ± 0.3 b	3.6 ± 0.3 c
Phenolic compounds	0.3 ± 0.1 a	0.4 ± 0.1 a	0.8 ± 0.4 a	ND	ND	0.3 ± 0.0

[a] Values, expressed as μg/L of 5-nonanol, are means ± standard deviations of three fermentations, each analyzed in duplicate. For each processing type, means in the same row labelled with different letters are significantly different ($P < 0.05$). ND = not detected.

Besides, the metataxonomy analysis of the fermenting brines (Fig. 2) revealed the presence of fungi of *Penicillium* genus after 7 months in Natural-style olives, although at low relative abundance (1.4%).

Two sulfur compounds, namely, dimethyl sulfide and dimethyl sulfoxide were found throughout fermentation in both olive types, with dimethyl sulfide being the predominant one.

Low concentrations of terpenes (mainly, linalool, α-terpineol and β-damascenone) were found in both olive types. In addition, volatile phenols were found in fairly small amounts. This disagrees with the results of previous studies with Spanish-style olives of the Manzanilla cultivar, where relatively high levels of phenolic compounds were detected, especially p-creosol (Cortés-Delgado et al., 2016; Sánchez et al., 2018; de Castro et al., 2019). This discrepancy may be due to the different origins of olives and differences in microbial growth during fermentation.

Based on the contents of volatile compounds and PCA, the samples were clearly separated according to sampling time in both olive types (Fig. 3). The volatile compounds mainly associated with samples at the first stage of fermentation were 2-pentanone and 2-pentanol in the Spanish style; and methyl butanoate, methyl isovalerate, acetoin, (E)-2-decen-1-ol, and styrene in the Natural style. Most volatiles were associated with the final stage of fermentation. Among them, we can highlight acetic acid, isopentanol, ethyl acetate, and ethyl lactate in the Spanish style; and 2-heptanone, 2-nonanone, methyl 2,5-dimethyl-3-furoate, and benzoic acid in the Natural style.

3.5. Correlation between microbiota and volatile compounds during fermentation

To elucidate the relationship between microbial communities and volatile compounds in the two processing styles, Spearman's correlations were calculated and heatmap diagrams were generated to visualize the correlation profiles (Fig. 4).

Focusing on the significant ($p < 0.05$) correlations for the dominant microbial taxa at the end of process, we can highlight that, in Spanish-style olives, *Lactobacillus* presented positive correlations with 37 volatile compounds, especially acids and esters such as acetic acid, butanoic acid, nonanoic acid, benzoic acid, methyl lactate, methyl acetate, ethyl acetate, ethyl 3-methylbutanoate, and ethyl 2-hydroxyisovalerat, all of them with correlation coefficients (ρ) greater than 0.9 (Fig. 5a); *Saccharomyces* showed positive correlations with 14 volatiles, of which octanoic and decanoic acids presented the highest correlations ($\rho \approx 0.9$); and *P. manshurica* showed positive correlations with 32 volatiles, of which butanoic acid, benzoic acid, isopentanol, and decanol presented the highest correlations ($\rho \geq 0.9$).

In case of Natural-style olives, *Aliidiomarina, P. manshurica,* and *Nakazawaea* showed similar correlation profiles including more than 30 positive correlations with various acids, alcohols, esters, ketones, terpenes and other compounds (Fig. 5b, left network). Among the highest correlations ($\rho > 0.9$) in common for these microbial taxa, we can highlight those with carbitol, ethyl phenylacetate, α-terpineol, phenol, and 2,3-dihydrobenzofuran. *Halomonas* and *Saccharomyces* showed correlation profiles totally different to those of *Aliidiomarina, P. manshurica,* and *Nakazawaea* (Fig. 5b, right network). The *Halomonas* genus presented positive correlations with 12 volatiles, of which 1-decanol showed the highest correlation levels ($\rho > 0.9$). *Saccharomyces* had positive correlations with 14 volatiles, of which (Z)-3-hexen-1-ol, 3-octanol, (Z)-3-hexenyl acetate and limonene showed the highest correlation values ($\rho > 0.9$).

It is worth to mention that various of the above-mentioned volatiles that showed significantly positive correlations were previously found as aroma-active compounds in green table olives [*viz.* nonanoic acid (waxy, cheesy, dairy), 1-heptanol (musty, leafy, herbal), 2-phenylethyl acetate (floral), limonene (greenery, fruity), α-terpineol (ferment)] (Iraqi et al., 2005) or in black table olives [*viz.* acetic acid (vinegar), 2-methylbutanoic acid (cheesy), isobutanol (ethereal, winey), isopentanol

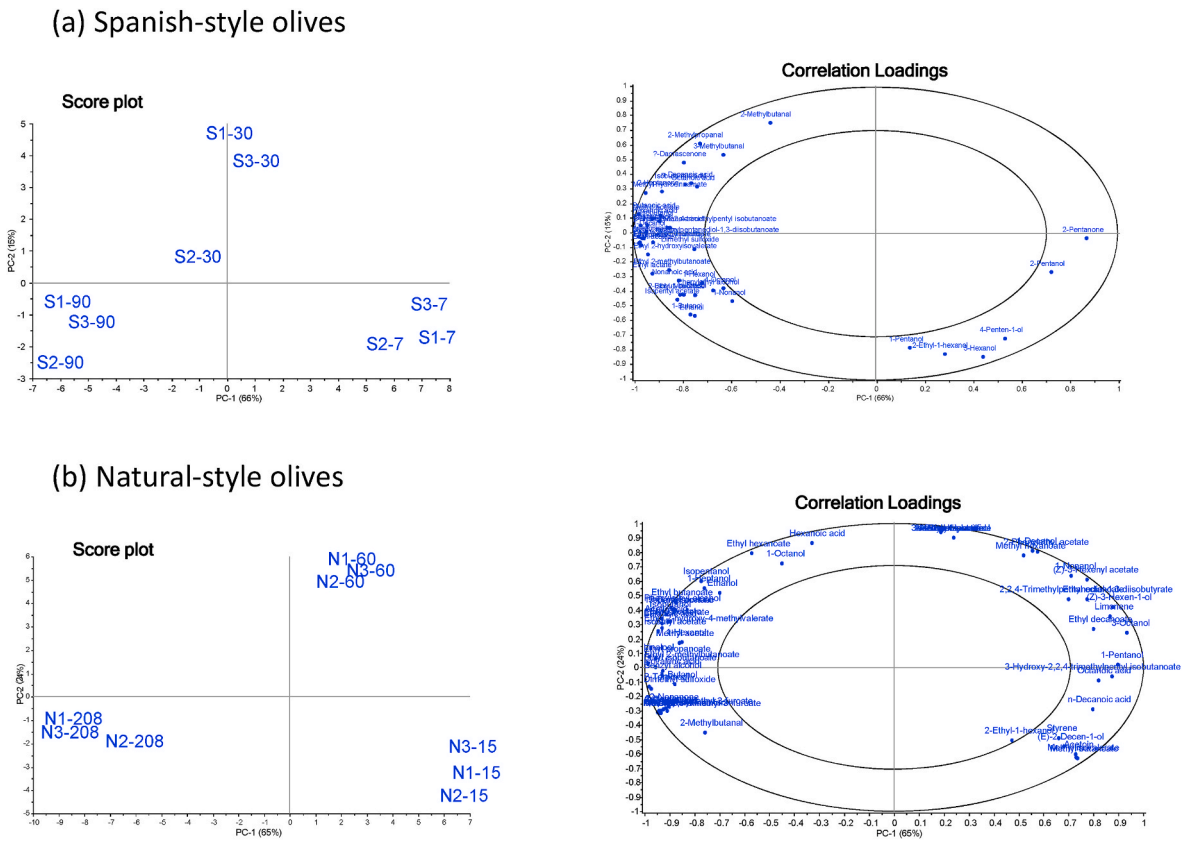


Fig. 3. PCA of volatile compounds during fermentation of Spanish-style and Natural-style green olives of the Manzanilla cultivar: (a) Spanish-style olives, S1, S2 and S3 refers to the three replicate fermentations studied; 7, 30 and 90 indicate the sampling time (days). (b) Natural-style olives. N1, N2 and N3 refers to the three replicate fermentations studied; 15, 60 and 208 indicate the sampling time (days). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

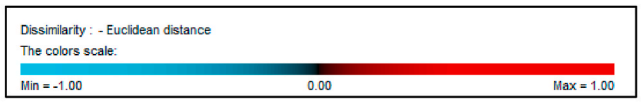
(fermented, alcoholic, banana), 1-hexanol (ethereal, alcoholic, fruity), (*Z*)-3-hexen-1-ol (green, flowery), phenylethyl alcohol (floral, sweet), ethyl propanoate (fruity, sweet), isopentyl acetate (fruity, sweet)] (Selli et al., 2018). Positive correlations for *Saccharomyces, P. manshurica*, and *Nakazawaea* with various of these volatiles would confirm the active role of yeasts in relation to table olive aroma, as widely accepted (Arroyo-López et., 2008; Penland et al., 2020). In this sense, Natural-style olives started with selected strains of the species *Saccharomyces cerevisiae* were found to produce higher levels of isopentanol and phenylethyl alcohol than those in spontaneous fermentations (Tufariello et al., 2019). More recently, a strain of the species *P. manshurica* was demonstrated to produce acetic acid, 2-methylbutanoic acid, and isopentanol as the major volatile compounds when this yeast was grown in an olive-derived culture medium; and a strain of the species *Nakazawaea molendinolei* mainly produced ethanol, ethyl acetate, phenylethyl alcohol, isobutanol, and (Z)-3-hexen-1-ol in the same culture medium (Montaño et al., 2021).

Bacterial communities such as *Lactobacillus* in Spanish-style olives as well as *Aliidiomarina* and *Halomonas* in Natural olives could also contribute to the aroma formation, as these bacteria showed strong correlations with some of the above-mentioned aroma-active compounds (e.g. acetic acid, nonanoic acid, and 1-heptanol with *Lactobacillus*; ethyl propanoate and α-terpineol with *Aliidiomarina*; 2-phenylethyl acetate with *Halomonas*). However, Benítez-Cabello et al. (2019) found that the inoculation of *Lactobacillus* strains, specifically *L. pentosus* and *L. plantarum,* as starters had little or even a negative impact on the formation of volatile compounds (e.g. 1-heptanol) in Spanish-style table olive fermentations.

The fact of some volatile compounds correlated with more than one microorganism would indicate that these compounds were possibly derived from highly connected metabolic pathways of a variety of microorganisms. In addition, it must be stressed that the correlation profiles for *Saccharomyces* and *P. manshurica*, which stood out in both olive types at the end of the processes, were clearly different in both olive types, possibly indicating interactions with other microbial communities during fermentation.

4. Conclusions

In our study, metataxonomy analysis and HS-SPME/GC-MS were used to investigate the dynamics of microbial and volatile compounds during fermentation of Spanish-style and Natural-style green table olives from Manzanilla cultivar, these two processes running in parallel. In this way, the effect of alkaline debittering pretreatment on the microbial communities growing during fermentation was revealed for the first time. Microbial profiles showed meaningful differences between the two types of table olives, which impacted their volatilome along with their physicochemical and biochemical characteristics. Regarding volatilome, the highest differences between the two olive styles were found for acids (higher content of acetic acid in Spanish-style olives) and carbonyl compounds (higher content of methyl ketones in Natural-style olives). In addition, the relationships between microbial communities and volatile compounds were revealed in each olive type, confirming the active role of yeasts and bacteria in relation to table olive aroma. Our findings demonstrate that LAB growth was not essential for a better volatile profile of the fermented product, although a comparative study of the sensory characteristics of the two olive styles was not carried out. The present study provides a better understanding of each fermentation process and may help the development of controlled fermentations using starter cultures of bacteria and/or yeasts for the production of high-



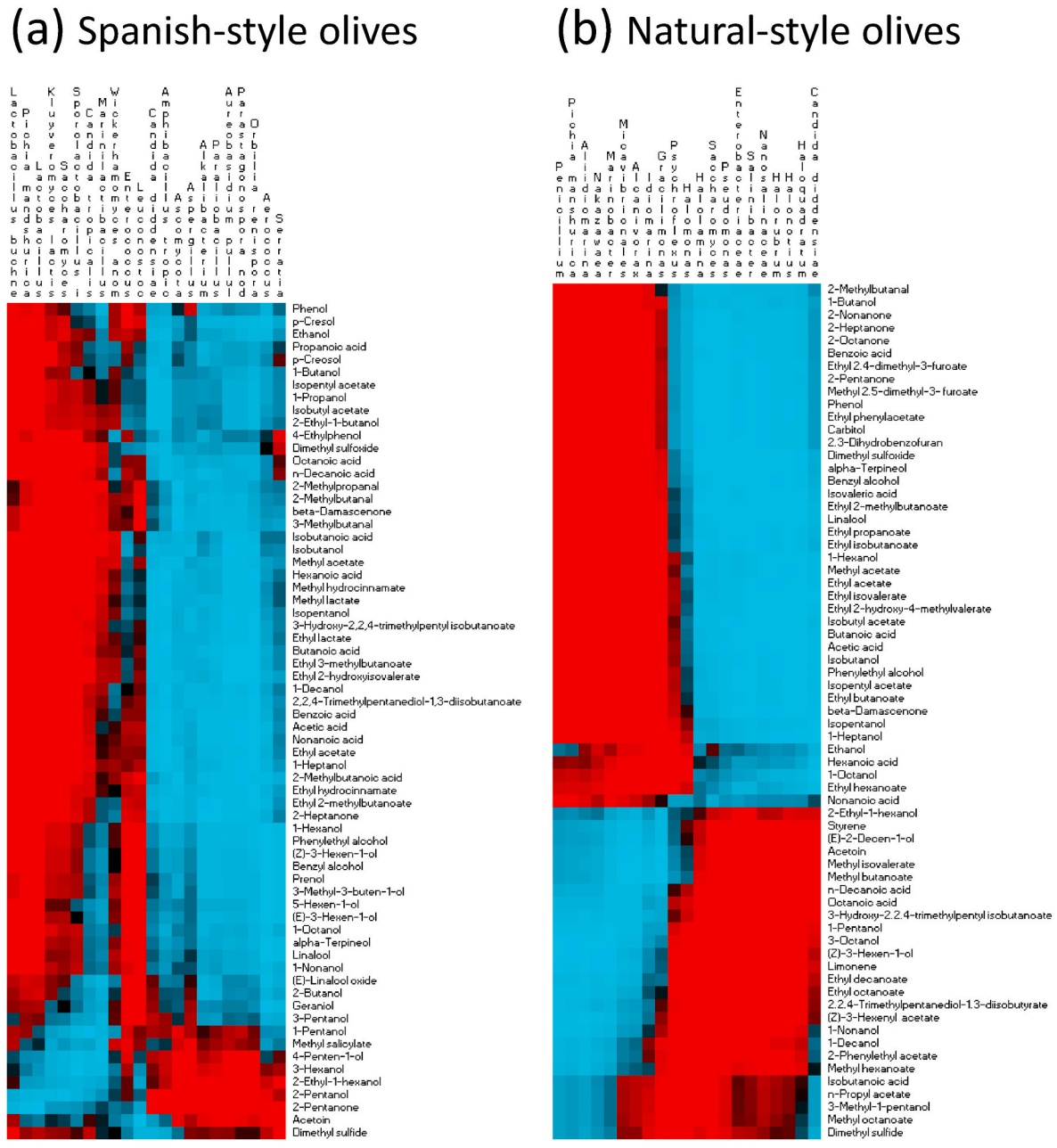


Fig. 4. Spearman's correlation between microbial OTUs and volatile compounds during fermentation. (a) Spanish-style olives, (b) Natural-style olives. Only microbial OTUs with abundances >1% in at least 2 samples were considered. Positive correlations are indicated in red while negative ones are indicated in cyan (see color scale at the top). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

quality green table olives of the Manzanilla cultivar.

Author contributions

Conceptualization, A.M and J.L.R.-B; Formal analysis, A.H.S., A.L.-L., J.L.R.-B, and A.C.-D.; 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., 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.

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Declaration of competing interest

The authors declare that they have no known competing financial

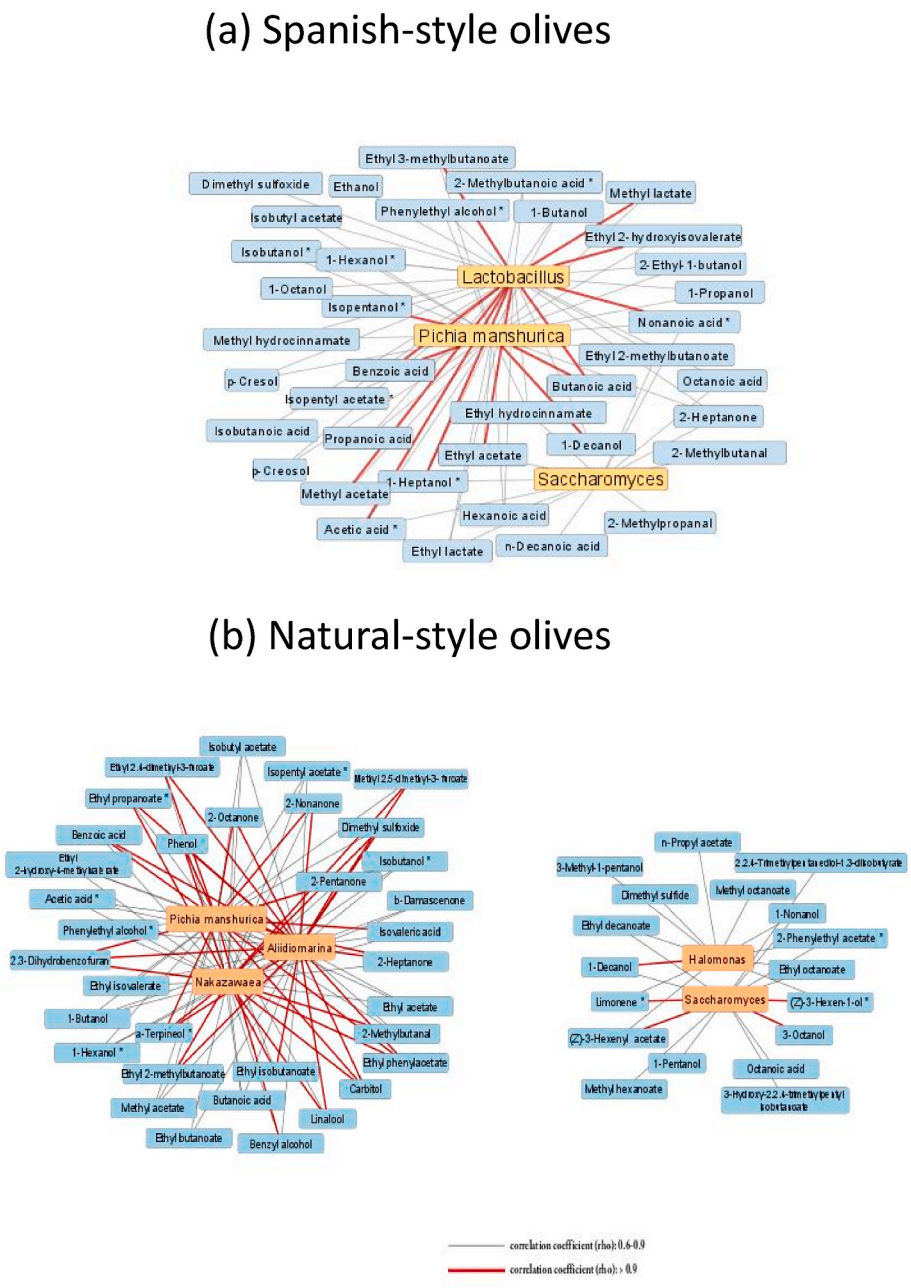

Fig. 5. Correlation network between the predominant microbial taxa at the final stage of fermentation and volatile compounds. (a) Spanish-style olives, (b) Natural-style olives. Only significant ($P < 0.05$) positive correlations are shown. The edges in red represent strong correlations levels ($\rho \geq 0.9$). The compounds marked with an asterisk have been previously reported as aroma-active compounds in table olives. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2023.104286.

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