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Brine salt concentration reduction and inoculation with autochthonous consortia: Impact on Protected Designation of Origin Nyons black table olive fermentations

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ABSTRACT

Nyons table olives, named after the French city where they are processed, are naturally fermented black table olives. Their specificity relies on the use of the "Tanche" olive variety harvested at full maturity and their slow spontaneous fermentation in 10% salt brine driven by yeast populations. This study aimed at investigating the benefit of inoculating autochthonous consortia to produce Nyons table olives by fermentation in 10% salt brine and in reduced salt conditions (8%). Two strategies were evaluated: inoculation with a defined autochthonous consortium and inoculation by spent brine backslopping. To define the consortium, yeasts were selected among 48 autochthonous isolates and key features included high halotolerance, low pectinolytic and proteolytic activities, however none had β-glucosidase activities. The consortium included eight yeast strains with distinct technological properties belonging to five dominant species, i.e. *Citeromyces nyonsensis*, *Pichia membranifaciens*, *Wickerhamomyces anomalus, Zygotorulaspora mrakii* and *Candida atlantica.* Fermentation trials were conducted over a year and compared by evaluating microbial community shifts (16S and ITS metagenetics) and volatile profiles (GC-MS). Regarding fermentations with the defined consortium, four out of five species implanted in early stages while one, *Pichia membranifaciens,* persisted and largely dominated by the end of the fermentation. Altogether, inoculation with the defined consortium did not disrupt microbial shifts compared to traditional fermentations although minor differences were observed in volatile profiles. The backslopping method yielded the highest impact on microbial populations and olive volatile profiles, with higher ester abundances at the end of fermentation. Finally, reduced salt in brine gave very promising results as no deleterious effects on microbial communities, volatile dynamics but also safety criteria of the olives were observed compared to traditional fermented olives.

1. Introduction

Table olives are among the most consumed fermented vegetables with 2.57 million tons produced in 2019 (International Olive Council, 2019). Although native to the Mediterranean region, they are consumed worldwide and appreciated for their pleasant taste as well as for their health benefits (Peres et al., 2017). Preparation of table olives relies on three main processes: Spanish-style process (lye-treated green olives prior to fermentation in brine), Californian-style preparation (ripe olives processing technology including lye treatment and air oxidation) and Greek-style olives (naturally fermented black olives in brine) (Romeo, 2012).

Nyons table olives benefit from the protected designation of origin (PDO) status. They are produced in southern France according to the Greek-style process and PDO specifications: Tanche variety olives are harvested at full maturity during the winter and directly immersed in

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10% sea salt brine for up to 1.5 years. Conventional and organic Nyons table olives microbiota and volatile profile evolution were recently deciphered during multiple year-long fermentations (Penland et al., 2020). As with many spontaneous or natural fermentations, rich and complex microbiota were revealed, in particular for fungal communities. The main fungal species identified were *Wickerhamomyces anomalus, Citeromyces nyonsensis, Zygotorulaspora mrakii, Pichia membranifaciens, Candida boidinii* while *Celerinatantimonas diazotrophica* was most abundant for bacteria. In this regard, Nyons table olives stand out as LAB play a minor role in fermentation while yeasts were shown to play a central role by contributing to both acidification and volatile profile enhancement (Coton et al., 2006; Penland et al., 2020).

The complex microbial diversity of naturally fermented table olives is most often correlated to complex organoleptic properties. However, natural fermentations can also lead to the presence of unwanted microorganisms and cause quality defects such as textural changes, off-flavors and off-odors (Lanza, 2013; Penland et al., 2021). In this context, the use of tailored starter cultures to better control fermentations is clearly of interest. With this objective, recent work has focused on the selection and inoculation of microorganisms with selected technological features and an emphasis on lactic acid bacteria (LAB) has been given (Campus et al., 2015; Panagou & Tassou, 2006). In recent years, some studies have also successfully explored the potential of yeast starters in table olive fermentations and beneficial outcomes regarding debittering and olive organoleptic profiles were observed (Ciafardini & Zullo, 2019; Psani & Kotzekidou, 2006). However, some yeast species metabolisms can also lead to olive spoilage, hence it is necessary to select strains with technological properties of interest. With this in mind, the most desirable selection criteria include halotolerance for growth in brine and acidification; β-glucosidase activity for phenolic compound degradation and debittering, and lipase/esterase activity to produce desirable aroma characters. On the other hand, undesirable properties correspond to strong pectinolytic and proteolytic activities or CO_2 production that may lead to textural and visual defects (Bevilacqua et al., 2012; Bonatsou et al., 2015; Corsetti et al., 2012).

Aside from the development of functional starter cultures, PDO black table olive fermentations could benefit from reduced sodium chloride content. Consumer's demands for healthy products have increased in recent years. While table olive nutritional properties (e.g. polyphenols, antioxidant properties) are well-known, their high sodium content may hinder frequent consumption. This is particularly true with naturally fermented black olives as high salt contents (up to 10%) are classically used to prevent spoilage and contribute to the debittering phase when compared to green olives (6%). Although recent studies have evaluated the substitution of NaCl with other chloride salts, e.g., KCl (Bautista Gallego et al., 2011; Zinno et al., 2017), such alternatives have to be accepted by both consumers and olive producers who are attached to traditional know-how, especially regarding PDO status products.

In this context, our study aimed at tailoring a consortium of autochthonous yeasts that mimic the natural diversity of Nyons table olives to better drive the fermentation in traditional and reduced salt conditions. Autochthonous yeast isolates were first screened for their main technological properties of interest. Then, two inoculation strategies were implemented: i) inoculation of an autochthonous undefined culture using brine backslopping method and ii) inoculation of a tailored yeast consortium in addition to the natural microbiota. Microbial dynamics and olive volatile profiles were evaluated in traditional 10% salt brine fermentations and in reduced salt condition (8% salt brine).

2. Material & methods

2.1. Yeast isolates & culture conditions

A total of 48 yeast isolates, representative of the microbiota identified in PDO Nyons table olive fermentations, were screened in the present study for their technological properties. Isolates were obtained from the first sampling dates (day 64 and 183 out of 460) of traditional natural Nyons table olive fermentations and were selected based on their highly heterogeneous profiles previously observed by FTIR clustering analyses (Penland et al., 2020). They were identified to the species level by sequencing the D1/D2 domain of the 26S rRNA gene (Kurtzman & Robnett, 1998).

2.2. Screening of technological characteristics

2.2.1. Culture conditions

Each isolate was grown on Yeast Glucose Chloramphenicol (YGC) agar supplemented with 5% NaCl for 5 days at 25 °C. Cultures for screening tests were prepared as follows. First, 10 mL of Tryptone Soy Broth with 0.6% yeast extract (TSBYE; tryptone soy 30 g/L, Glucose 2.5 g/L; yeast extract 6 g/L) were inoculated with one fresh colony and incubated for 48 h with agitation (120 rpm) at 25 °C. Then, 10 mL of fresh TSBYE broth were inoculated at 1% with the culture and incubated in the same conditions for 24 h in triplicate. Concentration was determined by counting yeast cells on Malassez cells (three cells were enumerated per replicate). Then, a standardized suspension at 10^6 CFU/mL was prepared for each replicate in physiological water and directly used as inoculum for the screening experiments.

2.2.2. Screening autochthonous yeast strains for technological properties

CO_2 production was evaluated using a protocol adapted from a conventional fermentation test for yeasts as described by Guinet (1985): 100 μL yeast suspension was inoculated in a tube containing 10 mL of TSBYE broth and a Durham bell. TSBYE was used as good growth of all tested isolates occured. It contained glucose as sole carbon source and yeast extract provided vitamins. Tubes were incubated for up to 8 days at 30 °C and checked daily for gas production. An isolate was considered to produce CO_2 when growth was clearly visible and the bell filled with gas raised to the top of the tube.

Salt tolerance was tested by inoculating each strain in TSB broth supplemented with sodium chloride (NaCl) to concentrations ranging from 0 to 10% in microplates. Each well was inoculated with 200 μL of broth and 20 μL of the isolate suspension to reach a final concentration of 10^5 CFU/mL. Microplates were incubated at 25 °C for 7 days. All microplates included control wells without inoculum to validate the experiments. Growth was monitored daily by observing the presence or absence of biomass in the wells: growth was considered negative when aspect was similar to the control, positive but weak (+) when a turbid suspension was visible, positive with good growth (++) when biomass or a cell pellet was clearly visible.

β-glucosidase, esterase, proteolytic, lipolytic, pectinolytic activities, hydrogen sulfide production and killer activities were qualitatively assessed using specific media as described below. For each test, the isolates to test were inoculated by depositing 5 μL of isolate suspension as a single spot on the medium surface.

β-glucosidase activity was assessed using arbutin as substrate as described by Bleve et al., (2015). Yeasts were considered β-glucosidase positive when a dark halo developed around the yeast colony.

Esterase and lipase activities were evaluated according to the methods described by Fadda et al., (2004). After incubation at 25 °C for 48 h, activities were considered positive when a halo could be seen around the yeast colony.

Proteolytic activity was assessed by depositing the yeast suspension on Plate Count Agar supplemented with 50% skim milk as described by Aponte et al., (2010). Plates were incubated at 22 °C for 5 days and proteolytic activity assessed by measuring the diameter of the opacified halo around the yeast colony.

Pectinolytic activity was studied as described by Hernández et al., (2007). Yeast Nitrogen base (0.67%) with 2% agar was used. The medium was then adjusted to pH 4 and supplemented with 1.25% pectin. After inoculation, plates were incubated for 10 days at 25 °C and pectinolytic activity was monitored daily by measuring the diameter of the

yeast colonies: activity was considered negative when no halo was observed, weak when the halo was inferior to 4 mm, strong when halo was superior to 5 mm.

Hydrogen sulfide production was assessed by depositing a yeast suspension on Biggy agar (Sigma-Aldrich, Germany) (Aponte et al., 2010). Plates were then incubated at 30 °C for 18 to 48 h. Isolates were considered H_2S-positive when the colonies displayed a dark brown color.

Killer activity of the different isolates was assessed using a confrontation test adapted from the protocol described by Woods and Bevan (1968). *Debaryomyces hansenii* CLIB 197 was used as a sensitive reference strain and inoculated as a surface mat on YGC agar supplemented with 0.3% methylene blue while yeast isolates were deposited as spots. Plates were then incubated for 5 days at 25 °C and checked daily. A killer activity was identified by a clear zone/blue killing zone around the tested yeast isolate spot.

2.3. Inoculated and spontaneous pilot-scale fermentation assays

2.3.1. Experimental design

Five fermentations were monitored over one year to study three experimental conditions (Fig. 1). All five were launched the same day with olives from the same variety (Tanche), caliber and batch. The five tanks were prepared according to the classical procedure: 300 kg of olives (from the same harvest) were immersed in 200 L of brine. Brine was prepared by mixing commercial sea salt (obtained by evaporating sea water) in water at 10% or 8% depending on the experimental condition. First, the impact of the tailored consortium on the traditional Nyons olive PDO process was assessed by inoculating the consortium in 10% sea salt brine (C10) and in the presence of autochthonous microorganisms. In parallel, a spontaneous fermentation in 10% brine (T10) corresponding to the traditional process, was also monitored and used as a control tank. The second experimental condition aimed at studying the impact of the same consortium in reduced-salt fermentation conditions. To do so, it was inoculated into a tank containing 8% salt (C8) while a spontaneous fermentation in 8% salt (T8) was also monitored as a control. The third experimental condition consisted in inoculating an undefined autochthonous consortium using the backslop method: fermented brine from a previous batch (inoculation rate 5%) was added to a 10% salt brine to perform this fermentation. Noteworthy, the batch used for backslopping was previously monitored in the study by Penland et al. (2020). For all fermentations, the microbiological and organoleptic characteristics were in accordance with PDO requirements and the Nyons olive cooperative specifications. For C8 and C10 tanks, the tailored consortium was directly added to the brine to obtain 5.10^7 CFU/L per strain final concentration (see in 2.3.2). All fermentation tanks were left untouched following immersion in brine and salt was not supplemented during the fermentation.

Fermentation progress was monitored by collecting samples in the tanks at day 1 (following inoculation) and during fermentation at days 8, 64, 167 and 365. At each sampling point and for each fermentation, a minimum of 500 g of olive and brine were sampled in sterile containers and stored at 12 °C until analysis. Samples were processed within 24 h and subjected to pH measurement, microbial population enumeration and microbial community shifts monitoring in brines using metagenetics and volatile profile changes over time.

2.3.2. Tailored yeast consortium preparation

The yeast consortium was prepared by mixing eight yeast strains (*Citeromyces nyonsensis* UBOCC-A-220066, UBOCC-A-220036, UBOCC-A-220035; *Pichia membranifaciens* UBOCC-A-220030, UBOCC-A-220048; *Wickerhamomyces anomalus* UBOCC-A-220016; *Zygotorulaspora mrakii* UBOCC-A-220020 and *Candida atlantica* UBOCC-A-220021), selected after the screening experiments. The consortium inoculated in C8 and C10 tanks was prepared as follows: each strain was grown on Potato Dextrose Agar supplemented with 5% NaCl for 5 days at 25 °C. Biomass was then gently scraped and resuspended in physiological water to obtain standardized suspensions of 2.10^8 CFU/mL for each strain. Each strain was then inoculated at final concentration of 5.10^7 CFU/L into the fermentation tanks.

2.3.3. Microbial monitoring by culture-dependent approach

2.3.3.1. Enumeration of microbial populations and isolations. Microbial populations were monitored for each tank during the year-long fermentation at days 1 (fresh fruits immersed in brine), 8, 64, 167 and

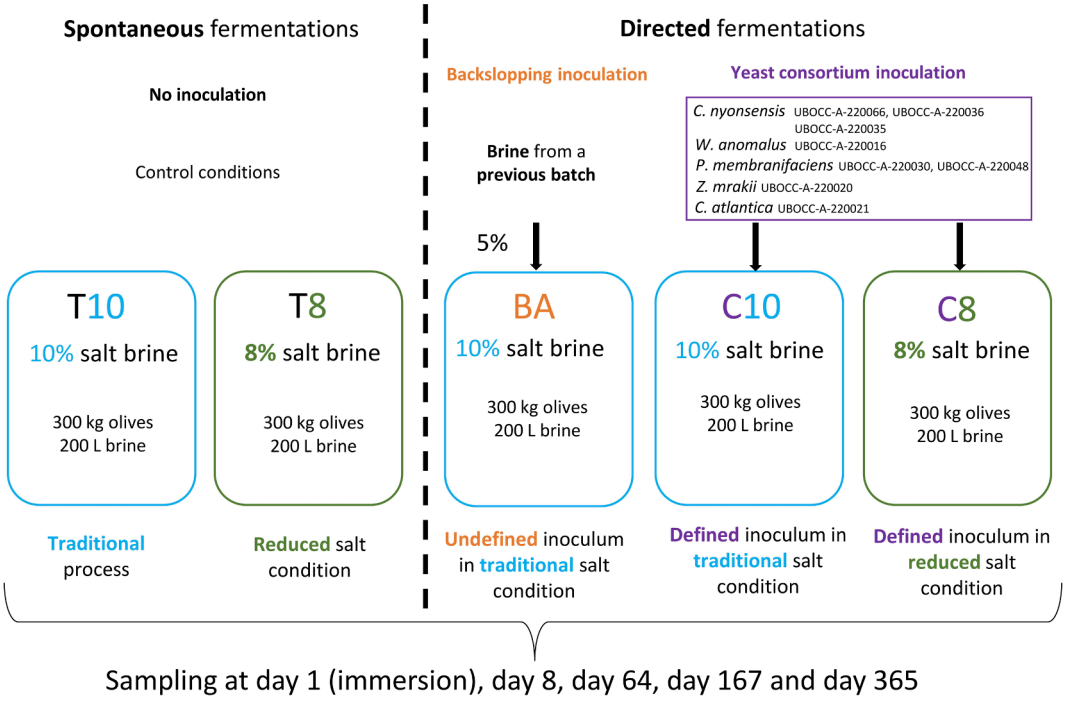

Fig. 1. Inoculation strategies used for the different Nyons table olive fermentations in the study.

365. For each sampling point, brine and olive fruits were aseptically mixed together: 12.5 g of olive flesh and 12.5 mL of brine were mixed with 225 mL Buffered Peptone Water and homogenized with a stomacher for 3 min at high speed. Seven microbial populations were enumerated: total fungal populations (Yeast extract Glucose Chloramphenicol medium (YGC, 25 °C, 5 days), halotolerant fungi (YGC + 5% NaCl, 25 °C, 5 days), total aerobic microbial populations (PCA, 30 °C, 72 h), halotolerant microbial populations (PCA+5% NaCl, 30 °C, 72 h), enterobacteria (VRBG, 30 °C, 48 h), lactic acid bacteria (LAB) (MRS+0.01% cycloheximide, 30 °C, 48 h; anaerobiosis) and halotolerant lactic acid bacteria (MRS+5% NaCl+0.01% cycloheximide, 30 °C, 48 h; anaerobiosis).

In order to verify that each individual strain developed and persisted in the inoculated fermentation tanks (C8 and C10), 50 colonies were randomly taken from YGC+5% NaCl plates at days 1, 64 and 167. All isolates (n = 200) were then purified on YGC+5% NaCl and cryopreserved in glycerol (15% w/w) and stored at −80 °C until M13-PCR fingerprinting.

2.3.3.2. M13 PCR fingerprinting of isolates in inoculated fermentations. M13 RAPD-fingerprinting was used to monitor strain persistence at days 64 and 167 of fermentation in the inoculated tanks C8 and C10. Genetic profiles were first generated for the eight inoculated strains using the method described by Guinebretiere and Nguyen-The (2003) with the M13 primer (5′-GAGGGTGGCGGCTCT-3′). They were then compared to those obtained for all yeast isolates from C8 and C10 tanks at the different dates. The repeatability and reproducibility of the experiments was evaluated by using the eight pure strains as controls in all M13-PCR experiments and verifying that no deviations in their genetic profiles occured. M13-PCR banding patterns were analysed and clustered using BioNumerics fingerprinting software version 5.1 (Applied Maths, Belgium).

2.3.4. Brine microbial communities monitored by metagenetic analysis

2.3.4.1. DNA extractions for metagenetic analyses. DNA was extracted from brine samples at days 1, 64 and 365 of fermentation. Extraction was performed in triplicates as described by Penland et al., (2020) using NucleoSpin Soil DNA kit (Macherey-Nagel, Germany). DNA extracts were stored at −20 °C until use.

2.3.4.2. 16S and ITS metagenetic analyses. DNA extracts were submitted to PCR amplification and high throughput sequencing to study bacterial and fungal diversity at the Genome Quebec sequencing platform (McGill university, Canada). For bacteria, V3-V4 region of the 16S rRNA gene was targeted using S-D-bact-0341-b-S-17 and S-D-BAct-0785-a-A-21 primers and using PCR conditions described by Klindworth et al. (2013). For fungi, the ITS3f/ITS4_Kyo1 primers targeting ITS2 region were used and PCR conditions were as described by Toju et al. (2012). ITS2 and V3-V4 amplicons were sequenced in the same run using Illumina Miseq PE300 technology generating 2x300 bp reads.

2.3.4.3. Bioinformatic and microbial diversity analyses. Sequences were pre-processed for quality and length using the following parameters: amplicon size between 370 and 490 bp for V3-V4 contigs and 100–530 bp for ITS2 contigs, mismatch rate was set at 0.1 and sequences with NNN were filtered out for both data types. Sequencing data were then analyzed using the FROGS pipelines as developed by Escudié et al. (2018) using Galaxy web-based platform (Afgan et al., 2018). Parameters and steps were as described in Penland et al. (2020). Regarding the final affiliation step, it was performed using SILVA (V138) and UNITE 8.2 fungi databases for 16S rRNA and ITS2 data, respectively. OTUs affiliated to contaminants such as chloroplasts or mitochondrial sequences in 16S rDNA data were excluded. When sequences were multi-affiliated by FROGS, because the targeted V3-V4 or ITS2 regions were unable to discriminate species, all the resulting putative species or genus were assigned into the final OTU table. In the end, 5,399,810 V3-V4 16S rRNA gene sequences and 4,651,870 ITS2 sequences were kept for diversity analysis. The OTU tables were rarefied to 72, 139 and 72,788 sequences for ITS data and 16S data, respectively. Based on rarefactions curves, both depth sequencing and normalization ensured good richness coverage (supplementary figure S2). Diversity and composition analyses were performed using Phyloseq and vegan packages under R software (McMurdie & Holmes, 2013). Alpha-diversity was estimated using Shannon index, while beta-diversity analyses were conducted using weighted UniFrac distances and visualized by Principal Coordinates Analysis (PCoA). PERMANOVA (999 permutations) was used to compare microbial community structures between tanks and assess the impact of the inoculation method and of the salt level on diversity.

2.3.5. Biochemical analyses

2.3.5.1. pH measurements in brine. Brine samples from each sampling point were analyzed for pH using a pH meter (Hanna Instruments HI 2020-02).

2.3.5.2. Olive volatile profile using GC-MS. Volatile profiles of olive fruits at day 1, 64 and 365 were obtained for each fermentation condition in triplicates using HeadSpace-GC-MS. Compound extraction was performed using a Perkin Elmer Turbomatrix HS-40 trap automatic headspace sampler with trap enrichment on 2.5 g +/− 0.1 g of olive fruits placed in 22 mL vials. Parameters and analyses were conducted according to methods previously described in Penland et al. (2020).

2.3.6. Statistical analyses

Statistical tests were performed using FactoMiner package under R software (Lê et al., 2008) and XLSTAT software (Addinsoft, Paris, France). Statistical analysis of GC-MS data was performed using a two-way analysis of variance (ANOVA) considering the different conditions and the stage of fermentation as variables. Tukey's post-hoc test was then used, in order to assess the differences among inoculated and natural fermentations. Hierarchical clustering and correlation analyses of the volatiles were performed with R Hmisc package (Harrell and Dupont, 2020) using Ward's minimum variance linkage and Euclidean distance method. All representations were obtained using R ggplot2 package (Wickham, 2016).

3. Results & discussion

3.1. Strain characterization and selection

LAB and yeast are responsible for fermentation *i.e.* mainly acidification and aroma coumpound production. However, the ratio between LAB and yeast is strongly dependent on the type of olive processing (Anagnostopoulos & Tsaltas, 2022). In Spanish-style processing, LAB are responsible for fermentation and lactic acid is the main acid produced. In natural style processing, like for Greek and Nyons olives, yeasts are predominant and mainly responsible for lower acidification as a result of acetic and citric productions and typical aroma compound production as previously described in Panagou et al. (2008), Bleve et al. (2015) and in Penland et al. (2020) for Greek and Nyons olives, respectively. Previous work showed that yeasts were the main microorganisms involved in Nyons table olive fermentations and five main species were identified: *W. anomalus*, *C. nyonsensis*, *Z. mrakii*, *C. boidinii* and *P. membranifaciens* (Penland et al., 2020). Among these species, a total of 48 autochthonous yeast isolates were selected and all isolates were screened for nine technological properties (Table 1).

Halotolerance and growth in brine are major criteria for strain selection. Indeed, such microorganisms must resist high osmotic stress induced by high salt levels encountered in brines, especially in the case

Table 1
Technological property screening of the autochthonous yeast isolates from Nyons natural black table olives. Symbols: "-" indicates an absence of activity; "+" indicates a positive although weak activity; "++" indicates a positive and strong activity.

Species (ITS region sequening)	Isolate[a]	Desirable properties								Undesirable properties			
		β -glucosidase	Killer activity	Salt tolerance (%)						Lipolysis	Proteolysis	Pectinolysis[b]	H_2S production	CO_2 production
				0	2	4	6	8	10					
Wickerhamomyces anomalus	R1C2D64.Y22	−	−	++	++	++	++	++	++	−	−	0.5	−	−
	R1C3D183.Y31	−	−	++	++	++	++	++	++	−	−	0.4	−	−
	UBOCC-A-220035	−	+	++	+	+	+	++	++	−	−	0.5	−	−
	R2B2D64.Y24	−	+	++	+	+	+	+	++	−	−	1.6	−	−
	R2C2D64.Y21	−	+	++	+	+	+	++	++	−	−	1.5	−	−
	R1C3D183.Y32	−	+	++	++	++	++	++	++	−	−	0.3	−	+
	R2B1D64.Y21	−	−	++	++	++	++	++	++	−	−	0.4	+	++
	R2C2D64.Y22	−	−	++	++	++	++	+	+	−	−	0.4	−	++
	R1C2D183.Y54	−	−	+	++	++	+	+	+	−	−	0.4	−	+
Pichia membranifaciens	R1B1D183.Y30	−	−	++	++	++	++	++	+	−	−	0.3	−	−
	R2B2D64.Y28	−	−	++	++	++	++	++	++	−	−	0.5	++	−
	UBOCC-A-220030	−	−	+	++	++	++	++	++	−	−	0.4	−	−
	R1C2D183.Y42	−	−	++	++	++	++	++	+	−	−	0.5	−	−
	R2B2D183.Y41	−	−	++	++	++	++	++	++	−	−	0.3	−	−
	R2C1D183.Y43	−	−	++	++	++	++	++	++	−	−	0.3	−	−
	R1C1D183.Y32	−	−	++	++	++	++	++	++	−	−	0.7	−	−
	UBOCC-A-220048	−	−	++	++	++	++	++	++	−	−	0.4	−	−
	R2C2D64.Y26	−	−	++	++	++	++	++	++	−	+	0.4	+	+
	R1B2D64.Y12	−	−	++	++	++	++	++	++	−	−	0.3	+	−
	R2B1D64.Y39	−	−	++	++	++	++	++	++	−	−	0.35	−	−
	R2B1D183.Y60	−	−	+	−	+	+	+	−	−	−	0	−	−
Citeromyces nyonsensis	R1B1D64.Y15	−	−	++	++	++	++	++	++	−	−	0	−	−
	R1C1D64.Y18	−	−	++	++	++	++	++	++	−	−	0.45	−	−
	R1C1D183.Y34	−	−	++	++	++	++	++	++	−	−	0.45	−	−
	R2B2D64.Y34	−	−	++	++	++	++	++	++	−	−	0.3	−	−
	R2C1D64.Y22	−	−	++	++	++	++	++	++	−	−	0.3	−	−
	R2B3D183.Y50	−	−	++	++	++	++	++	++	−	−	0.3	−	−
	R2C3D183.Y59	−	−	++	++	++	++	++	++	−	−	0.35	−	−
	R2C2D183.Y59	−	−	++	++	++	++	++	++	−	−	0.35	−	−
	R1C1D64.Y17	−	−	++	++	++	++	++	++	−	−	0.3	−	−
	R1C3D183.Y40	−	−	++	++	++	++	++	++	−	−	0.3	−	−
	R2B3D64.Y32	−	−	++	++	++	++	++	++	−	−	0.4	−	−
	UBOCC-A-220066	−	−	++	++	++	++	++	++	−	−	0.3	−	−
	UBOCC-A-220036	−	−	++	++	++	++	++	++	−	−	0.3	−	−
	R1B1D64.Y12	−	−	++	++	++	++	++	++	−	−	0.3	−	−
	R1B2D183.Y28	−	−	++	++	++	++	++	++	−	−	0.3	−	−
	UBOCC-A-220035	−	−	++	++	++	++	++	++	−	−	0.5	−	−
Candida boidinii	R2B3D183.Y55	−	−	++	++	++	++	+	−	−	+	0.5	++	−
	R1C2D183.Y41	−	−	++	++	++	++	+	−	−	−	0.5	++	+
	R1B1D64.Y8	−	−	++	++	++	++	++	+	−	−	0.5	++	−
	R1B3D183.Y24	−	−	++	++	++	++	++	−	−	−	0.7	−	−
	R1B2D64.Y24	−	−	++	++	++	++	++	++	−	−	0.3	−	−
Candida atlantica	**UBOCC-A-220021**	−	−	++	++	++	++	++	++	−	−	0.3	−	−
Zygossacharomyces mrakii	R2C2D64.Y38	−	−	++	++	++	++	++	++	−	−	0.4	−	−
	UBOCC-A-220020	−	−	++	++	++	++	++	++	+	−	0.4	−	−
	R2C3D64.Y38	−	−	++	++	++	++	++	++	−	−	0.4	−	−
	R1B2D183.Y40	−	+	+	++	++	++	+	+	−	−	0.3	−	−
	R1C1D64.Y21	−	−	++	++	++	++	++	++	−	−	0.4	−	−
Strong (%)		0	10	92	99	91	90	85	80	0	0	25	8	4
Weak (%)		0	0	8	6	9	10	15	12	2	4	71	4	8
Negative (%)		100	90	0	2	0	0	0	8	98	96	4	88	88

[a] Isolates in bold were selected to form the complex culture.
[b] Haloexpressed in cm as mean value triplicates; proteolysis activity considered negative when halo was < 0.3 cm; "weak" when halo was between 0.3 and 0.5 cm; "positive and strong" for halo > 0.5 cm.

of PDO Nyons table olives requiring 10% salt. Moreover, salt levels can fluctuate throughout the fermentation in relation with diffusion and evaporation. Thus, although all strains were isolated from black olive fermentations in brine, their salt tolerance up to 10% was re-evaluated. Results showed inter and intra-species level variations. While most isolates (>85% of total isolates) grew well in 0–8% NaCl, 79% showed good growth at 10%. The isolates that did not grow at 10% NaCl belonged to *C. boidinii* and *P. membranifaciens*.

Various enzymatic activities also play a major role in olive fermentations and may originate from either the olives or be of microbial origin. β-glucosidase activity is particularly of interest as it hydrolyses oleuropein (Ghabbour et al., 2011; Johnson & Mitchell, 2018). Oleuropein is the major component responsible for the bitter taste in olive fruits and is known to disappear throughout fermentation, making olives edible. Here, no activity was detected among the 48 screened isolates. β-glucosidase activities were reported in multiple studies for different yeasts species and were shown to be strain-dependent in *C. boidinii* (Bautista Gallego et al., 2011; Ciafardini et al., 2006). When considering the species involved in this fermentation, this finding contrasts with previous research that described high β-glucosidase activity among *W. anomalus* isolates (Bautista Gallego et al., 2011). These results confirmed what we previously observed (Penland et al., 2020). Nyons table olive fermentations were characterized by a slow debittering process, and no correlations between oleuropein decrease and microbiota composition were identified. Ramírez et al. (2016) and Johnson and Mitchell (2018) reported alternative ways to microbial oleuropein degradation in olives such as endogenous hydrolysis and chemical hydrolysis leading to hydroxytyrosol, elenolic acid and glucose release. Hence, oleuropein diffusion from pulp to brine, chemical hydrolysis in acidic conditions and/or endogenous enzymes from olive fruits are the most likely mechanisms involved in the debittering process and sugar release rather than yeast or bacteria activity. Moreover, these mechanisms are reported to happen slowly which corroborates with the unusually long fermentation. In a similar way, no esterase and lipase activities (except for *Z. mrakii* R2B3D64.Y38) were observed among tested isolates. These activities, linked with lipolysis, are desirable as they can improve olive flavor by inducing amino acid catabolism and the release of fatty acids, which are known ester precursors.

Many enzymatic activities are responsible for negative organoleptic impacts and olive quality defects. High pectinolysis and proteolysis can result in excessive softening of olive fruits (Golomb et al., 2013). Regarding proteolysis, only two isolates, belonging to *P. membranifaciens* and *C. boidinii,* showed weak *in vitro* activity. Concerning pectinolytic activity, strong variations were observed as 25% and 72% of isolates showed strong or weak activities, respectively. High pectinolytic activities were mainly observed among *W. anomalus* and *C. boidinii* isolates while weak activities were observed for *C. nyonsensis* and *Z. mrakii*. Yeast metabolism can also indirectly lead to off-flavors or defects in olive fermentations (Lanza, 2013). CO_2 release is a consequence of sugar consumption and inherent to fermentation. However, if released at too high levels, CO_2 can accumulate and form gas-pockets under the skin and/or provoke brown discoloration patches in the olive flesh known as the “fish-eye” defect (Vaughn et al., 1972). Sulfur compounds are commonly found in fermented olives and participate to flavor. Hydrogen sulfide production can also be produced by yeasts via sulfur-containing amino acid catabolism (Huang et al., 2017). However, too high H_2S levels are linked with unpleasant off-flavors and considered as a quality defect (Aponte et al., 2010). In this regard, the ability of yeast isolates to produce excessive amounts of H_2S and/or CO_2 were assayed for all isolates. No production was observed for both activities for 41 out of 48 isolates. H_2S production was mainly observed in *C. boidinii* while weak CO_2 production was identified for four *W. anomalus* isolates.

Finally, killer activity was also evaluated. This activity reflects the production of toxic proteins or glycoproteins that can hamper the growth of other sensitive yeast strains (Hernández et al., 2008). Overall, four isolates belonging to the *W. anomalus* species (10.4%) presented inhibition activity. Yeasts with killer activities belonging to *W. anomalus* and also *Saccharomyces cerevisiae*, *P. membranifaciens* have already been isolated from table olives (Belda et al., 2017; Marquina et al., 1992). Our findings thus corroborate these results. Some authors considered killer activity as undesirable although it can provide a competitive advantage for a strain to persist during fermentation. Recent work also discussed its potential to control both fungal populations and spoilage microorganisms (Arroyo López et al., 2012; Mannazzu et al., 2019).

Based on these results, the yeast consortium was constituted by excluding all strains with strong undesirable activities. Then, in order to maximise and reflect the natural diversity, and based on desirable activities, eight strains with distinct phenotypes were selected within the four dominant species: *W. anomalus* UBOCC-A-220016, chosen for its killer activity and lack of CO_2 production, *P. membranifaciens* UBOCC-A-220030, UBOCC-A-220048 and *C. nyonsensis* UBOCC-A-220066, UBOCC-A-220036 UBOCC-A-220035 chosen for their halotolerance and low pectinolytic activities and *Z. mrakii* (n = 1). One *C. atlantica* strain (UBOCC-A-220021) presenting an interesting salt tolerance profile was also selected. As no *C. boidinii* isolate presented a suitable profile for fermentation, the species was not kept for the tailored consortium. These five species already have Generally Recognized As Safe (GRAS) status and constituted the complex cultures used for controlled fermentation assays.

3.2. Microbial populations and pH changes in brines during inoculated (BA, C) and control fermentations (T)

In each tank, the main microbial populations were monitored at five time points during fermentation (Fig. 2). Fungal and total aerobic counts reached similar levels by the end of fermentation in all experimental conditions. Focusing on fungi, after yeast consortium inoculation and brining on day 1, populations were 10-times higher in inoculated tanks, C10 and C8, compared to control tanks, T10 and T8. For the backslopping condition (BA), initial fungal populations were ~1.8 log CFU/g higher than the other conditions, as expected, given the fact that high fungal populations were already present in spent brines.

Initial pH in brines ranged from 5.54 to 5.95 depending on the condition. However, the same acidification profile was observed for all five fermentations. Within the first week, pH dropped by 0.3 up to 0.6 units, then slowly decreasedto pH 4.4 at day 64 and remained stable until the end of fermentation (Fig. 2). Based on these results, neither the yeast consortium nor reduced salt content had an impact on acidification.

The initial pH drop was concomitant with a 1 log decrease in microbial populations in T8 and T10 fermentations. This change is most likely related to the plant-associated microorganisms and filamentous fungi species that disappeared in this high salt environment. By day 64, similar fungal populations were reached, ~5 log CFU/g, for all fermentations. Populations then slightly increased by 0.5 log CFU/g until day 167 and stabilized up to day 365 for T10 and BA conditions, while they decreased to reach 4.6 log CFU/g in the C10 condition.

The same trends were observed in the reduced salt fermentations. Populations slightly increased by 0.3–0.5 log CFU/g between days 64 and 167, then increased to 4.8 and 4.5 log CFU/g by day 365 in C8 and T8 tanks, respectively. These values were approximately 1 log lower than T10 and BA conditions at the same stage. Interestingly, in 8% salt fermentations, lower halotolerant fungal populations were observed compared to total fungal counts.

Lactic acid bacteria populations were below the detection limits in all fermentations. This corroborates our previous results (Penland et al., 2020). This is probably due in part to the high salt content in brines. In the present study, LAB were still not detectable even under 8% reduced-salt conditions. Similar findings were also observed by Tassou et al. (2002). They showed that 8% salt concentration delayed LAB appearance during black table olive fermentations.

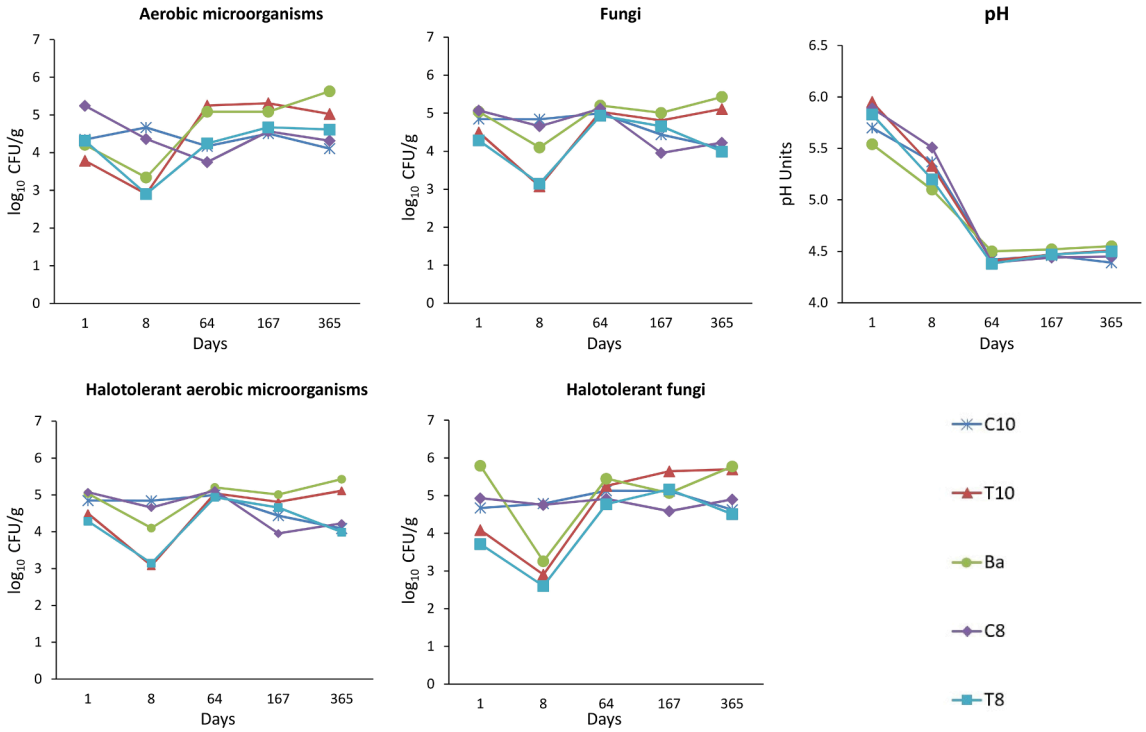

Fig. 2. Microbial population counts (log CFU/g) during Nyons table olives inoculated and spontaneous fermentations. Tanks: C10 fermentation inoculated with yeast consortium in 10% salt brine – T10 spontaneous fermentation in 10% salt brine – C8 fermentation inoculated with yeast consortium in 8% salt brine – T8 spontaneous fermentation in 8% salt brine BA – fermentation inoculated by backslopping in 10% salt brine.

In addition, all fermentations were analyzed for safety and hygiene criteria according to European regulation n° 2073/2005 at day 365. Criteria included enterobacteria, coagulase-positive staphylococci, *E. coli* population counts as well as absence of foodborne pathogens (*Listeria monocytogenes* and *Salmonella* spp.). All criteria were satisfied (data not shown), thus, addition of the yeast consortium or reduced salt content (8% brine) did not jeopardize the safety and microbial quality of the final product.

3.3. Fungal community composition of inoculated (BA and C) and spontaneous fermentations (T).

In order to determine whether the inoculated yeast consortium persisted during fermentation, M13-RAPD-PCR was applied on selected isolates from the two directed fermentations (C8, C10). This method was previously used for table olive yeast genotyping (Benítez-Cabello et al., 2016; Tofalo et al., 2013). In the present case, genetic profiles were obtained for the eight isolates belonging to the yeast consortium. Within a given species, only slight differences in RAPD patterns were observed between isolates despite their distinct technological characteristics; these were compared with those obtained from the C8 and C10 tank isolates (**Supplementary Fig. S3**). As a consequence, intra-specific discrimination of the inoculated species could not be done by M13-RAPD so this method was rather used to evaluate persistence of the inoculated species. Results for each tank over time were expressed as the percentage of isolates with the same genetic profiles as the inoculated species (Table 2). These data were used to strengthen metagenetic analysis results.

ITS2 amplicon sequencing was used to investigate differences in fungal communities over time and between experimental conditions. Beta-diversity analysis based on weighted UniFrac distance explained 96.3% of the variance and mostly separated samples by fermentation stage (Fig. 3**.A**). However, neither salt rate nor inoculation significantly impacted fungal communities (PERMANOVA test $P < 0.05$) except on day 1. Following brining, as expected, fungal communities strongly differed depending on the inoculation condition as shown in Fig. 3**.B**. At day 1, C8 and C10 tanks reflected the inoculation of *C. nyonsensis, P. membranifaciens, W. anomalus* and *Z. mrakii*, while in spontaneous fermentations (T8 and T10 tanks), fungal communities were dominated by *Aureobasidium pullulans* (abundance > 75%). *A. pullulans* is a yeast-like fungi often isolated from fruit. Its presence was confirmed by cultural methods at day 1 while completely absent from day 8 samples (data not shown). By day 64, the impact of the tailored consortium tended to decrease as all fermentations harbored similar fungal communities with dominance of *Z. mrakii* and *P. membranifaciens* although at various abundances. *Z. mrakii* represented more than 70% of relative abundance in C8 and C10 tanks at day 64. This result was the main difference observed with M13-PCR results where this species was subdominant and only represented 2 and 6% of the isolates in C8 and C10 tanks at the

Table 2
Species implantation in inoculated tanks C8 and C10 based on M13-PCR fingerprints of yeast isolates. Values are given as percentages of isolates with the same genetic profiles as the inoculated species (based on the total number of isolates collected at the sampling point).

Species	Tank					
	C10			C8		
% of isolates	day 1	day 64	day 167	day 1	day 64	day 167
Citeromyces nyonsensis	37.5	10	24	37.5	28	8
Wickerhamomyces anomalus	12.5	18	–	12.5	–	–
Zygotorulaspora mrakii	12.5	6	–	12.5	2	–
Pichia membranifaciens	25	66	36	25	66	36
Candida atlantica	12.5	–	–	12.5	–	–
NP1 (later identified as *Candida boidinii)*	–	–	40	–	–	20
NP2	–	–	–	–	–	4
NP3	–	–	–	–	–	2
NP4	–	–	–	–	–	2

NP - New profile.

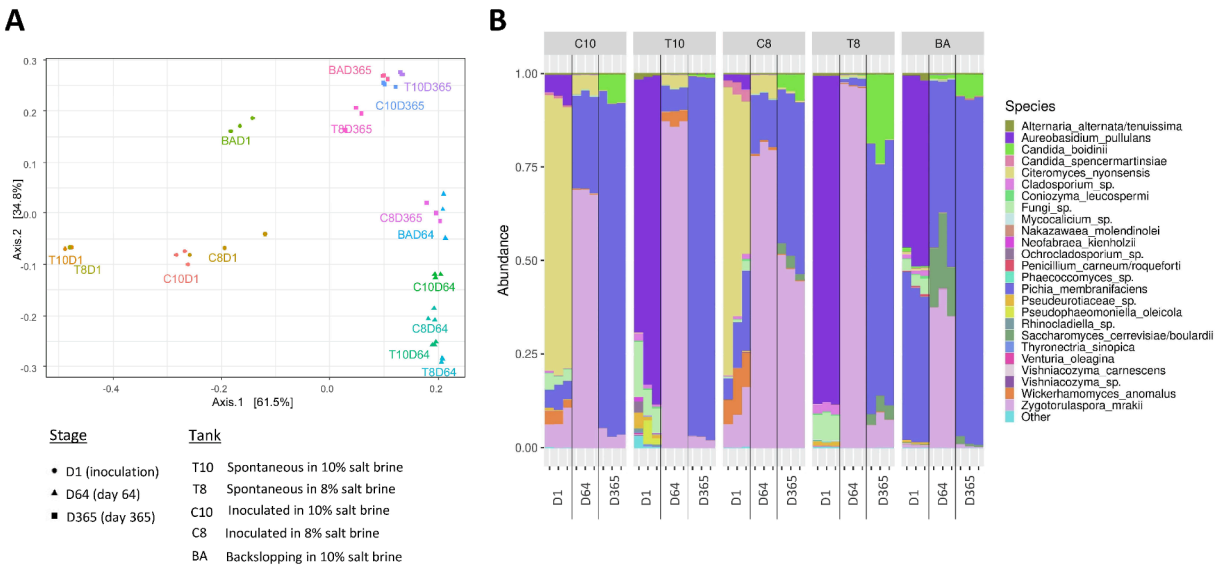

Fig. 3. Fungal community structure and dynamics during spontaneous and inoculated Nyons table olive fermentation using ITS2 metagenetic analysis. (A) Principal Coordinate Analysis based on weighted UniFrac distance (B) Fungal community composition during fermentations. Day 1 corresponds to the sampled brine following inoculation of the consortium (C10 and C8 tanks) or spent brine (BA tank).

same stage, respectively. Besides isolation biases, it might indicate that *Z. mrakii* is present at very low levels or in a viable but not cultivable state at day 64 which is coherent with the observed dynamic changes during fermentation. *P. membranifaciens* dominance strengthened over fermentation time: it represented 10 and 25% at day 64 in C8 and C10 respectively, and reached near 50 and 90% relative abundance by day 365. This was corroborated by M13 results as, by day 167, *P. membranifaciens* already dominated and represented over half of all tested isolates in both C10 and C8 fermentations. Noteworthy, *P. membranificiens* was found at higher abundances in directed fermentations compared to natural ones. Both *W. anomalus* and *C. nyonsensis* decreased during fermentation, all conditions considered: *W. anomalus* was no longer detected at day 64, while *C. nyonsensis* was still detected by M13-PCR at day 167 but no longer at day 365. Finally, *C. atlantica* was an exception and was the only inoculated species that did not colonize the brine. Indeed, this species was only identified by M13-PCR on day 1 and was not detected by metagenetics. This result was unexpected as it was selected for its high tolerance to salt and acidity. It might be inferred that the inoculation rate was too low for the species to persist at the beginning of the fermentation. We can also suppose that competition-exclusion or antagonism with other species could occur, leading to the disappearance of *C. atlantica.*

Except on day 1, similar trends were observed with the backslopping technique indicating that addition of spent brine at the start of fermentation did not impact fungal community dynamics. Concerning fungal diversity on day 1, a co-dominance of *A. pullulans* and *P. membranifaciens* was observed. This result was expected as *P. membranifaciens* was the most dominant yeast at the end of the fermentation used as a backslop (Penland et al, 2020).

P. membranifaciens dominance in all conditions demonstrated that this species is particularly well-adapted to the fermentation conditions in the early process stages as previously observed by Pereira et al. (2015). Moreover, this yeast species was able to persist despite the salt rate and acidity changes throughout fermentation. According to Kurtzman (2011), some *Pichia membranifaciens* strains are able to utilize acids such as citric and lactic acids which might explain its persistence. In the case of *W. anomalus,* its prevalence decreased after day 64 which was consistent with what was previously observed (Penland et al., 2020) despite the fact that *W. anomalus* UBOCC-A-220016 displayed killer properties. This result suggests that this strain was not active against other dominant members of the fungal microbiota or that this activity was not expressed during fermentation. Concerning the latter hypothesis, several studies showed that environmental factors such as pH and NaCl percentage could impact yeast killer activity (Llorente et al., 1997). Regarding NaCl effect, Hernández et al. (2008) observed that killer activity of indigenous *W. anomalus* isolates from olive brine increased at 8% compared to 5% but decreased at 10% NaCl. However, in this study, no such effect was observed regarding the reduced salt fermentation.

Altogether, salt reduction only seemed to temporarily favor *C. nyonsensis* growth (observed at day 64), as higher abundances were observed in inoculated fermentations at 10% compared to 8% salt for all inoculated isolates, including *W. anomalus*, based on M13-PCR monitoring and metagenetic community analysis. Interestingly, *C. boidinii* was detected at the end of all fermentations although at higher abundances in reduced salt conditions compared to traditional control ones (T10). This result may be explained by a decrease of salt-level in brine by diffusion into the olive fruits during the last stages of fermentation.

These results, combined with the species dynamics observed in reduced salt conditions, raise questions about the environmental factors that allow yeast strains to maintain themselves or not during fermentation. Other criteria should also be investigated such as tolerance to specific acids or phenolic compounds that are present in high concentrations during the fermentation such as oleuropein. The latter compound was moreover shown to exhibit antifungal activity and to inhibit growth of various microorganisms during olive fermentations (Zorić et al., 2016).

3.4. Bacterial community shifts during inoculated and spontaneous fermentations using 16S metagenetic analysis

Bacterial community composition during the different fermentation trials was also investigated by metagenetics and compared through beta-diversity analysis. Similarly to fungi, bacterial communities differed mostly according to the fermentation time on axis 1 (59.7% of variance, $P < 0.001$) but also according to salt rate on axis 2 (37.1% of variance, $P < 0.005$) (Fig. 4.**A**). Indeed, 8% fermentations (T8 and C8) were clearly opposed on axis 2 to 10% salt brine condition (C10 and T10) at day 1 of fermentation. Composition analyses at the species-level (Fig. 4.**B**) revealed that C10 and T10 conditions presented a significantly higher bacterial diversity than C8 and T8 conditions with similar abundances of marine bacteria genera (e.g., *Alcanivorax, Salinicola*, *Marinobacter*, *Halomonas*, *Methylophaga*). Meanwhile, 8% salt fermentation tanks were

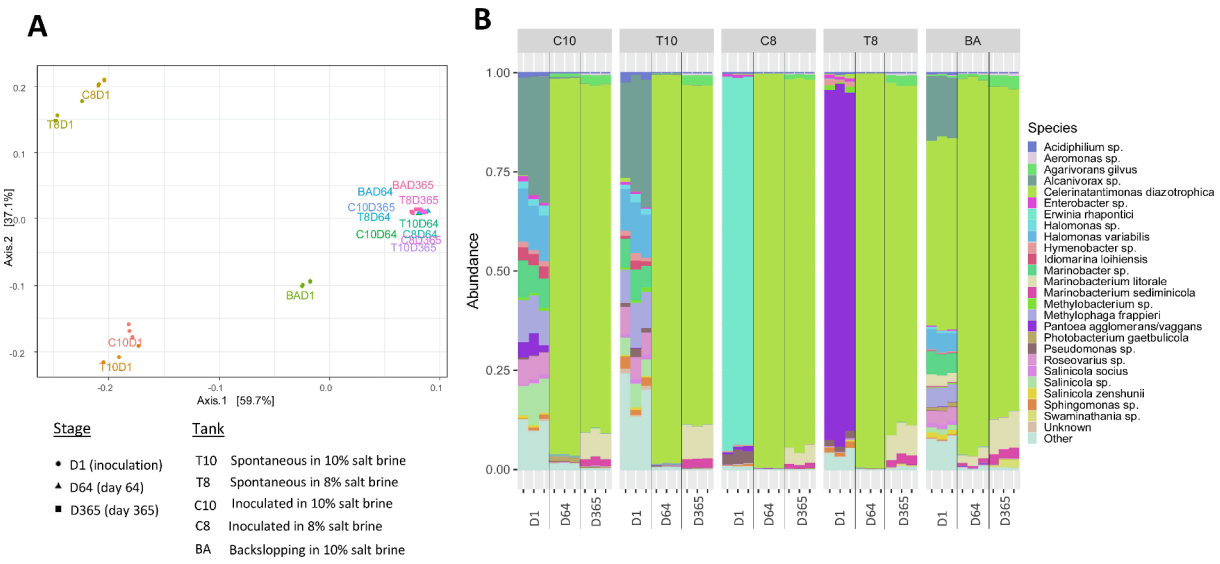

Fig. 4. Bacterial community structure and dynamics during spontaneous and inoculated Nyons table olive fermentation using 16S rRNA gene V3-V4 region metagenetic analysis. (A) Principal Coordinate Analysis based on weighted UniFrac distance (B) Bacterial community composition during fermentations. Day 1 corresponds to the sampled brine following inoculation of the consortium (C10 and C8 tanks) or spent brine (BA tank).

dominated by two plant-associated Enterobacteriaceae: *Pantoea agglomerans* (ex- *Erwinia herbicola*) and *Erwinia rhapontici* in T8 and C8 tanks, respectively. High abundance of marine bacteria was expected as commercial sea salts are used for brine preparation (Kazou et al., 2020; Medina et al., 2016). Likewise, *Erwinia* spp. most likely diffused from the fresh olive fruit to the brine and persisted when a lower salt rate was used. Thus, a 10% salt rate most likely operates a selection on bacterial communities in the early days of fermentation as Enterobacteria were detected in lower abundances in 10% brine compared to 8% brine. However, population levels must be very low and their viability weak since, according to the cultural microbiological analysis, no enterobacteria were enumerated in these samples. Moreover, as fermentation progressed, these species were no longer detected (Fig. 4**.A)**. Samples from all tanks at days 64 and 365 were grouped and their bacterial compositions were indeed similar with communities dominated by *Celerinatantimonas diazotrophica* (relative abundance over 90%) although diversity slightly increased by the end of fermentation with higher abundances of *Marinobacter* spp. This bacterium was previously identified as dominant at the end of Nyons table olive fermentations. As expected, it dominated the "backslopping" fermentation community after brining as it was already present in the spent brine used as an inoculum. These results indicate that bacterial community was only temporarily impacted by salt reduction and not affected by yeast inoculation. This also suggests that other factors drive bacterial community shifts during this fermentation process.

3.5. Volatile profiles changes during directed and spontaneous fermentations

The impact of the different fermentation conditions on the olive volatile profiles was investigated at three stages (days 1, 64 and 365). A total of 59 volatile compounds were identified (Table 3**)**. They mostly corresponded to esters (n = 21), fatty acids (n = 8), alcohols (n = 8), aldehydes (n = 8), ketones (n = 6), phenols (n = 3) and others classes (n = 5). Differences between volatile profiles were mostly related to fermentation stages rather than fermentation conditions (Fig. 4). Volatile kinetics were similar regardless of the condition: the start of the fermentations was correlated with highest abundances of aldehydes that rapidly and significantly decreased between days 1 and 64. During this phase, alcohol abundances increased as well as some esters such as ethyl acetate, ethyl propanoate and ethyl 2-methylpropanoate. However, most esters and phenol compounds significantly increased between days 64 and 365. These dynamics are well correlated to previous findings on Nyons table olives (Penland et al., 2020) and other natural olive fermentations (Bleve et al., 2014; De Angelis et al., 2015; Randazzo et al., 2017).

Differences between the fermentation conditions were only noticeable at day 365 based on hierarchical clustering results (Fig. 5). Backslopping fermentation (BA) stood out as samples were clearly separated from the others. Furthermore, clustering also separated C8 and T8 from C10 and T10 samples, highlighting a strong impact of the reduced salt condition. Inoculated and natural fermentations with the same salt level shared closer profiles which is not surprising considering that their microbial composition and dynamics were comparable. Nevertheless, differences in individual compound abundances were found between the different fermentation conditions (Supplementary **Fig. S4**).

The backslopping method (BA tank) led to significantly higher abundances of seven compounds when compared to the natural fermentation tank T10, namely 3-methylbutanol, 2-phenylethanol, pentan-3-one, 4-ethylphenol, ethyl 3-methylbutanoate, ethyl octanoate and styrene ($P < 0.05$). Noteworthy, the effects of backslopping were higher than those observed with the tailored consortium as nine compounds were identified at higher abundances (BA tank vs C10 tank). The impacted volatile compounds were also different from the ones observed in the inoculated fermentation C10.

The use of a tailored consortium led to different outcomes depending on salt content. Inoculation in 10% salt brines led to significantly higher abundances of six compounds (hexan-1-ol, 4-ethylphenol, 2-methoxyphenol, ethyl benzoate, ethyl octanoate and styrene) and lower abundances of two compounds (propanoic acid and ethyl propanoate). In 8% inoculated olives, higher abundances of seven compounds (ethanol, 2-methoxyphenol, ethyl acetate, ethyl butanoate, ethyl oct-7-enoate, hexyl acetate and styrene) and lower abundances of four compounds (propanoic acid, pentan-3-one, ethyl 3-methylbut-2-enoate and ethyl propanoate) were observed when compared to the respective control fermentation conditions ($P < 0.05$). Compounds mostly belonged to esters in both cases although different compounds were impacted. Noteworthy, inoculation of the tailored consortium systematically and positively impacted the abundances of 2-methoxyphenol and styrene, and negatively impacted propanoic acid and ethyl propanoate. 2-Methoxyphenol (also known as guaiacol) is a marker of black olive fermentations while styrene is also frequently identified as part of table

Table 3
Volatile compounds identified by HS-GC-MS during directed and spontaneous fermentations of Nyons table olives.

Family	Compound	Pubchem CID	EIC (*m/z*)	LRI [a]	Identification [b]	Odor descriptor [c]
Alcohols	Ethanol	702	45	903	DB, LRI, S	Alcoholic
	Propan-1-ol	1031	59	1029	DB, LRI, S	Alcoholic
	2-Methylpropan-1-ol	6560	74	1097	DB, LRI	Winey
	3-Methylbutanol	31,260	70	1208	DB, LRI	Fermented, fruity, pungent
	Hexan-1-ol	8103	56	1363	DB, LRI	Green, fruity, pear
	(E)-hex-2-en-1-ol	5,318,042	82	1409	DB, LRI	Fruity, green
	Phenyl methanol	244	108	–	DB	Floral, rose, phenolic
	2-Phenylethanol	6054	91	–	DB	Floral, sweet, rosery
Aldehydes	2-Methylpropanal	6561	72	799	DB, LRI, S	Cooked-caramel
	3-Methylbutanal	11,552	57	871	DB, LRI, S	Malty
	Hexanal	6184	56	1064	DB, LRI, S	Fatty, green, leafy
	2-Heptanone	8051	74	1167	DB, LRI	Fruity, green banana
	Octanal	454	84	1278	DB, LRI	Fatty, green
	Nonanal	31,289	98	1382	DB, LRI	Fatty, waxy
	Benzaldehyde	240	106	1507	DB, LRI, S	Almond
	2-Phenylacetaldehyde	998	91	1630	DB, LRI, S	Honey, floral rose
	4-Propylbenzaldehyde	120,047	148	–	DB	–
Esters	Methyl acetate	6584	74	812	DB, LRI	–
	Ethyl acetate	8857	61	834	DB, LRI	–
	Ethyl propanoate	7749	102	926	DB, LRI	Fruity, winey
	Ethyl 2-methylpropanoate	956	116	943	DB, LRI	Ethereal, fruity
	Ethyl butanoate	7762	71	972	DB, LRI, S	Sweet, fruity
	Ethyl 2-methylbutanoate	24,020	57	1039	DB, LRI	Sweet, fruity
	Ethyl 3-methylbutanoate	7945	115	1045	DB, LRI	Sweet, fruity
	2-Methylpropyl 2-methylpropanoate	7351	101	1079	DB, LRI	Fruity, banana
	Methyl 3-methylbuten-2-oate	13,546	83	1104	DB, LRI, S	–
	Ethyl 3-methylbut-2-enoate	3,014,021	128	1216	DB, LRI	–
	Ethyl hexanoate	31,265	88	1227	DB, LRI, S	Sweet, fruity
	Hexyl acetate	8908	84	1266	DB, LRI	Fresh, sweet, floral, green
	Ethyl 2-hydroxypropanoate	92,831	75	1336	DB, LRI	Fruity
	Ethyl octanoate	7799	101	1425	DB, LRI	Fruity, winey
	Ethyl oct-7-enoate	544,127	101	1476	DB, LRI	–
	Ethyl nonanoate	31,251	101	1525	DB, LRI	Fruity, rose, rum, wine
	Ethyl benzoate	7165	150	1653	DB, LRI	Mild, fruity, cooked
	Ethyl 2-phenylacetate	7590	91	1776	DB, LRI	Sweet, floral honey
	Methyl 3-phenylpropanoate	7643	104	–	DB	Honey, fruity, wine
	Ethyl 3-phenylpropanate	16,237	91	–	DB	Phenolic, woody
	Ethyl 3-phenylprop-2-enoate	7649	131	–	DB	Fruity, balsamic
Fatty acids	Acetic acid	176	60	1433	DB, LRI, S	Pungent, overripe fruit
	Propanoic acid	1032	74	1532	DB, LRI, S	Acidic, dairy, fruity
	4-Hydroxybutanoic acid	10,413	42	1612	DB, LRI	–
	Butanoic acid	264	73	1621	DB, LRI, S	Acidic, buttery
	2-Methylbutanoic acid	20,653	74	1663	DB, LRI, S	Acidic, buttery
	Hexanoic acid	8892	73	–	DB, LRI, S	Fruity, fatty sour
	Octanoic acid	379	101	–	DB, LRI, S	Oily, rancid, capric
	Nonanoic acid	8158	73	–	DB	Fatty
Ketones	Pentan-3-one	7288	86	952	DB, LRI	Ethereal
	Butane-2,3-dione	650	86	963	DB, LRI	Buttery
	3-Hydroxybutan-2-one	179	88	1273	DB, LRI, S	Sour milk
	1-Hydroxypropan-2-one	8299	74	1288	DB, LRI	Sweet, caramellic
	6-Propyloxan-2-one	12,777	99	–	DB	Coconut
Phenols	1,2-Dimethoxybenzene	7043	138	1719	DB, LRI	Spicy, vanilla
	2-Methoxyphenol	460	109	–	DB	Phenolic, woody
	Phenol	996	94	–	DB	Phenolic
	4-Ethylphenol	31,242	107	–	DB	Phenolic, smoky
Others	Methylsulfonylmethane	1068	62	733	DB, LRI	Sulfury
	Styrene	356	78	1244	DB, LRI	Sweet, balsam, floral
	Methylsulfinylmethane	679	63	1551	DB, LRI	Fatty, oily, salty
	α-Farnesene	5,281,516	93	1740	DB, LRI	Woody, green

[a] LRI = Linear Retention index - calculated based of retention time of n-alcane mixture injected in the column.
[b] Identification performed based the following criteria: RI—comparison of LRI calculated with data published in the literature and in intern; DB—comparison of mass spectral data with those of NIST 2008 library; and S—comparison of LRI with those of authentic standards injected in the same GC-MS system.
[c] odor descriptor associated with the compounds on The goodscentscompany database (http://www.thegoodscentscompany.com).

olive volatilomes (Bleve et al., 2015; de Castro et al., 2018). Styrene production from cinnamic acid or 2-phenylethanol was demonstrated to be carried out by many yeast species including *Pichia* and *Candida* spp. The higher abundances in inoculated fermentations is thus logical. Lower abundances of propanoic acid and its derivative ester, ethyl propanoate, is an interesting outcome. Moreover, propanoic acid abundances were also significantly lower in the 8% spontaneous fermentation (T8 tank) than in the 10% spontaneous fermentation (T10). In table olives, propanoic acid, which is an intermediary compound in branched amino acid catabolism (Gonzalez-Garcia et al., 2017), is usually considered as a marker of olive spoilage and related to "zapateria" defect resulting in off-odors production (de Castro et al., 2018). Indeed, it is related to propionibacteria outgrowth when salt levels and pH are too high. It was thus unexpected to be associated with the reduced salt assay. Moreover, no off-odors related to this defect were detected by the local producer.

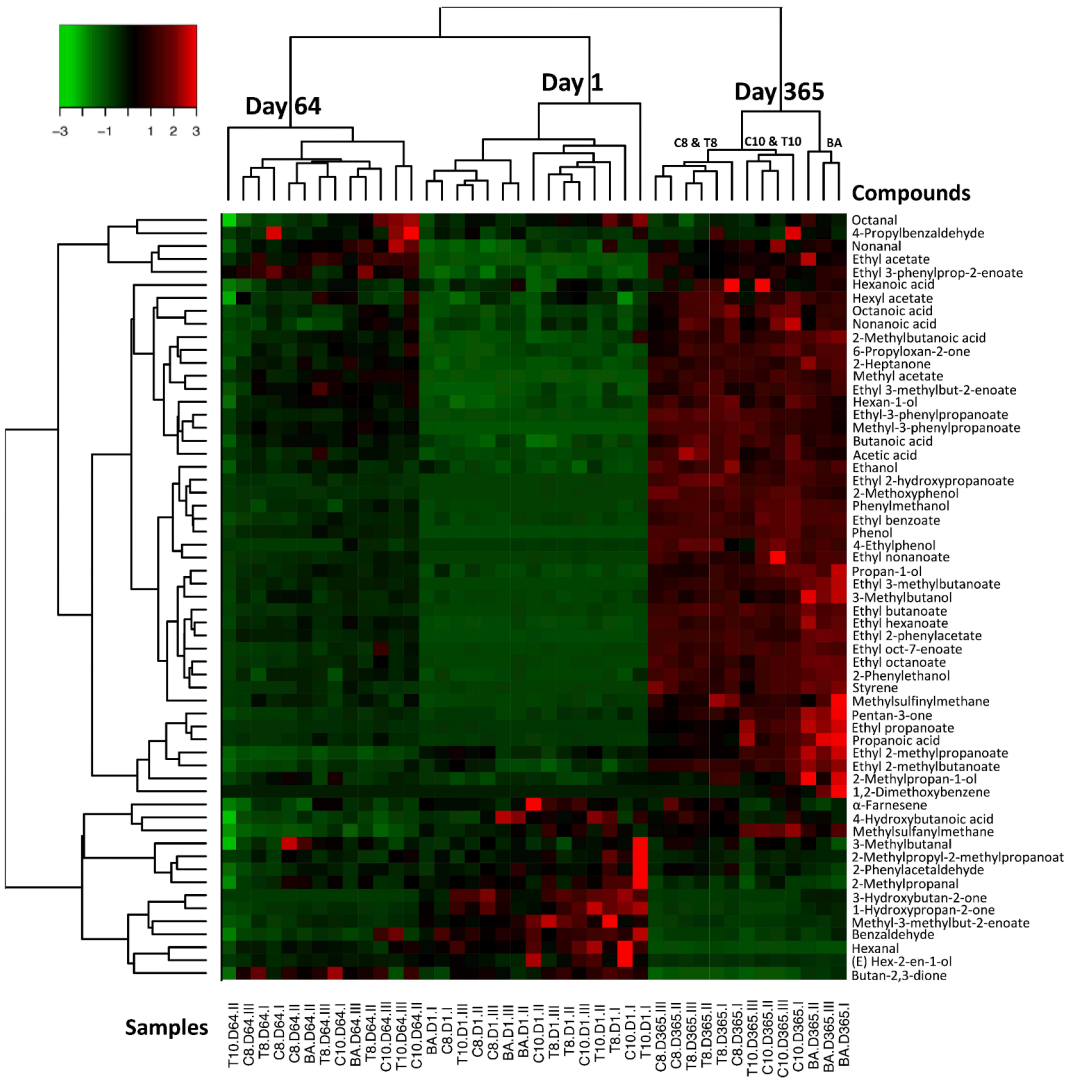

Fig. 5. Normalized heat-map of volatile abundances changes during inoculated and spontaneous (control) Nyons olives fermentations. Hierarchical clustering was performed using Ward's linkage method and Euclidian distance. Samples are displayed at the bottom while volatile compounds are displayed on the right part of the map. Color green to red shows low to high normalized abundances. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Reducing salt levels from 10 to 8% had a limited impact on volatile abundances (Supplementary Fig. S4, C8 versus C10 and T8 versus T10). In addition to propanoic acid and ethyl propanoate abundances, lower abundances of 1,2-dimethoxybenzene and higher abundances of 4-ethylphenol and ethyl 2-methylpropanoate were observed at 8% salt. Differences between salt levels were more pronounced in the presence of the tailored consortium. Six compounds were found in lower abundances at day 365 in 8% brine fermentations compared to 10% ones (ethyl benzoate, ethyl propanoate, 1,2-dimethoxybenzene and methylsulfonylmethane).

Altogether and regardless of the inoculation method (consortium or backslopping), the directed fermentations resulted in richer ester abundances. Such outcomes were previously observed in olive fermentation inoculated with yeasts and/or lactic acid bacteria (Bautista Gallego et al., 2011; Bleve et al., 2015; Pino et al., 2018). Esters are known to bring fruity and flowery aromas and higher abundances could enhance pleasant aromas of Nyons table olives. However, it has to be pointed out that research focusing on odor thresholds in fermented beverages showed that specific esters, related with pleasant aromas such as ethyl acetate, could lead to off-odors if overly abundant (de la Roza et al., 2003). The balance of aroma compounds therefore needs to be considered and sensory attributes evaluated. In this regard, preliminary sensory evaluations were carried out by the producer which included a panel of three experts. Olives with 8% and 10% salt were appreciated while olives fermented using the backslopping method were negatively perceived due to a mild but "acetic" flavor (data not shown). These first findings need to be strengthened by more thorough sensory analyses in the future.

Overall, inoculations by backslopping or with the consortium and salt reduction led to different olive volatile profiles after a year-fermentation. This might be surprising considering fermentation monitoring revealed no significant shifts in microbial communities or acidification trends at the end of fermentation (day 365). Nevertheless, it is interesting to notice that the backslopping conditions, which differed the most in initial microbial communities (day 1), was the one with the most different volatile profile in the end. It can thus be inferred that intermediary microbial community shifts were missed in the current experimental design and that those led to the observed differences in volatile profiles. Moreover, similar acidification trends following pH would only reflect global acidity evolution and not individual organic acid changes or other metabolic shifts such as amino acid ones. It is well known that organic acids and amino acids can be used by yeasts as

precursors to volatile compounds during food fermentations (Fairbairn et al., 2017; Montaño et al., 2021). As a consequence, salt content and minor (not statistically significant) changes in early fermentation stages can still impact the microbial metabolism and the volatile profile without disrupting the global microbial dynamics in the end.

4. Conclusion

The present study aimed at defining a tailored yeast consortium based on the beneficial traits of individual strains and to evaluate its use in natural Nyons table olive fermentations. The consortium consisted of eight autochthonous yeast strains belonging to the five-dominant species. Key features of strains included high halotolerance, low pectinolytic and proteolytic activities but no strains presented β-glucosidase activities. Fermentation with this tailored consortium was compared to both backslopping and natural fermentation conditions. The impact of reduced salt (from 10 to 8% NaCl) in brine was also evaluated.

Fermentation outcomes demonstrated a high resilience of fungal and bacterial communities. Regardless of the conditions, the same fungal species' dominances and shifts were observed by the end of fermentation. The backslopping method demonstrated the highest impacts on microbial populations and olive volatile profiles, with higher ester abundances at the end of fermentation compared to both the defined complex cultures and spontaneous fermentations used as reference conditions. However, preliminary sensory analyses by the local producer underlined an overall lack in aromas and a pronounced mild acetic taste. The fermentations with the autochthonous tailored consortium had less impact on both microbial and volatile profiles. No major differences were detected during the preliminary tasting sessions by the local producer indicating that an autochthonous starter culture can be used to carry out this fermentation. Finally, reduced salt in brine gave very promising results. No deleterious effects on microbial counts, volatile dynamics or safety criteria of the olives were observed. Olives produced in 8% brines were the most appreciated by the tasting panel. Considering fermentation trials were performed using one tank per condition, these results should be confirmed for other harvest periods by supplementary fermentations to verify reproducibility. If confirmed, these results are thus promising for future reduced salt fermentations of Nyons table olives without altering its natural microbial diversity and characteristics. This is also clearly in line with current consumer demands for traditional, tasty products as well as national nutritional policies aiming at reducing salt intake in the general population.

Author contribution

MC, JM and SMD obtained the funding and supervised the study. MC, JM and MP designed the experiments. MP and AP performed the experiments and analyzed the data. MM and SD provided technical assistance for GC-MS and microbiological analyses respectively. MP drafted the manuscript. MC, JM, HF and SMD edited and proofread the manuscript. All authors contributed to the article and approved the present version.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111069.

References

Afgan, E., Baker, D., Batut, B., van den Beek, M., Bouvier, D., Cech, M., Chilton, J., Clements, D., Coraor, N., Grüning, B. A., Guerler, A., Hillman-Jackson, J., Hiltemann, S., Jalili, V., Rasche, H., Soranzo, N., Goecks, J., Taylor, J., Nekrutenko, A., & Blankenberg, D. (2018). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses : 2018 update. *Nucleic Acids Research, 46*(W1), W537–W544. https://doi.org/10.1093/nar/gky379

Anagnostopoulos, D. A., & Tsaltas, D. (2022). Current Status, Recent Advances, and Main Challenges on Table Olive Fermentation: The Present Meets the Future. *Frontiers in Microbiology, 12*.

Aponte, M., Ventorino, V., Blaiotta, G., Volpe, G., Farina, V., Avellone, G., Lanza, C. M., & Moschetti, G. (2010). Study of green Sicilian table olive fermentations through microbiological, chemical and sensory analyses. *Food Microbiology, 27*(1), 162–170. https://doi.org/10.1016/j.fm.2009.09.010

Arroyo López, F. N., Romero Gil, V., Bautista Gallego, J., Rodriguez Gomez, F., Jimenez Diaz, R., García García, P., Querol Simon, A., & Garrido Fernandez, A. (2012). Potential benefits of the application of yeast starters in table olive processing. *Frontiers in Microbiology, 3*. https://doi.org/10.3389/fmicb.2012.00161

Bautista Gallego, J., Arroyo López, F. N., Romero Gil, V., Rodríguez Gómez, F., García García, P., & Garrido Fernández, A. (2011). Chloride salt mixtures affect Gordal cv. Green Spanish-style table olive fermentation. *Food Microbiology, 28*(7), 1316–1325. https://doi.org/10.1016/j.fm.2011.06.002

Belda, I., Ruiz, J., Alonso, A., Marquina, D., & Santos, A. (2017). The Biology of Pichia membranifaciens Killer Toxins. *Toxins, 9*(4), 112. https://doi.org/10.3390/toxins9040112

Benítez-Cabello, A., Bautista-Gallego, J., Garrido-Fernández, A., Rantsiou, K., Cocolin, L., Jiménez-Díaz, R., & Arroyo-López, F. N. (2016). RT-PCR–DGGE Analysis to Elucidate the Dominant Bacterial Species of Industrial Spanish-Style Green Table Olive Fermentations. *Frontiers in Microbiology, 7*. https://doi.org/10.3389/fmicb.2016.01291

Bevilacqua, A., Corbo, M. R., & Sinigaglia, M. (2012). Selection of Yeasts as Starter Cultures for Table Olives : A Step-by-Step Procedure. *Frontiers in Microbiology, 3*. https://doi.org/10.3389/fmicb.2012.00194

Bleve, G., Tufariello, M., Durante, M., Grieco, F., Ramires, F. A., Mita, G., Tasioula-Margari, M., & Logrieco, A. F. (2015). Physico-chemical characterization of natural fermentation process of Conservolea and Kalamàta table olives and developement of a protocol for the pre-selection of fermentation starters. *Food Microbiology, 46* (Supplement C), 368–382. https://doi.org/10.1016/j.fm.2014.08.021

Bleve, G., Tufariello, M., Durante, M., Perbellini, E., Ramires, F. A., Grieco, F., Cappello, M. S., De Domenico, S., Mita, G., Tasioula-Margari, M., & Logrieco, A. F. (2014). Physico-chemical and microbiological characterization of spontaneous fermentation of Cellina di Nardò and Leccino table olives. *Frontiers in Microbiology, 5*, 570. https://doi.org/10.3389/fmicb.2014.00570

Bonatsou, S., Benítez Cabello, A., Rodriguez-Gómez, F., Panagou, E., & Arroyo-López, F. (2015). Selection of yeasts with multifunctional features for application as starters in natural black table olive processing. *Food Microbiology, 46*, 66–73. https://doi.org/10.1016/j.fm.2014.07.011

Campus, M., Sedda, P., Cauli, E., Piras, F., Comunian, R., Paba, A., Daga, E., Schirru, S., Angioni, A., Zurru, R., & Bandino, G. (2015). Evaluation of a single strain starter culture, a selected inoculum enrichment, and natural microflora in the processing of Tonda di Cagliari natural table olives : Impact on chemical, microbiological, sensory and texture quality. *LWT - Food Science and Technology, 64*(2), 671–677. https://doi.org/10.1016/j.lwt.2015.06.019

Ciafardini, G., & Zullo, B. A. (2019). Use of selected yeast starter cultures in industrial-scale processing of brined Taggiasca black table olives. *Food Microbiology, 84*, Article 103250. https://doi.org/10.1016/j.fm.2019.103250

Ciafardini, G., Zullo, B. A., & Iride, A. (2006). Lipase production by yeasts from extra virgin olive oil. *Food Microbiology, 23*(1), 60–67. https://doi.org/10.1016/j.fm.2005.01.009

Corsetti, A., Perpetuini, G., Schirone, M., Tofalo, R., & Suzzi, G. (2012). Application of starter cultures to table olive fermentation : An overview on the experimental studies. *Frontiers in Microbiology, 3*. https://doi.org/10.3389/fmicb.2012.00248

Coton, E., Coton, M., Levert, D., Casaregola, S., & Sohier, D. (2006). Yeast ecology in French cider and black olive natural fermentations. *International Journal of Food Microbiology, 108*(1), 130–135. https://doi.org/10.1016/j.ijfoodmicro.2005.10.016

De Angelis, M., Campanella, D., Cosmai, L., Summo, C., Rizzello, C. G., & Caponio, F. (2015). Microbiota and metabolome of un-started and started Greek-type fermentation of Bella di Cerignola table olives. *Food Microbiology, 52*, 18–30. https://doi.org/10.1016/j.fm.2015.06.002

de la Roza, C., Laca, A., García, L. A., & Díaz, M. (2003). Ethanol and ethyl acetate production during the cider fermentation from laboratory to industrial scale. *Process Biochemistry, 38*(10), 1451–1456. https://doi.org/10.1016/S0032-9592(03)00026-8

de Castro, A., Sánchez, A. H., López-López, A., Cortés-Delgado, A., Medina, E., & Montaño, A. (2018). Microbiota and Metabolite Profiling of Spoiled Spanish-Style Green Table Olives. *Metabolites, 8*(4). https://doi.org/10.3390/metabo8040073

Escudié, F., Auer, L., Bernard, M., Mariadassou, M., Cauquil, L., Vidal, K., Maman, S., Hernandez-Raquet, G., Combes, S., & Pascal, G. (2018). FROGS : Find, Rapidly, OTUs with Galaxy Solution. *Bioinformatics (Oxford, England), 34*(8), 1287–1294. https://doi.org/10.1093/bioinformatics/btx791

Fadda, M. E., Mossa, V., Pisano, M. B., Deplano, M., & Cosentino, S. (2004). Occurrence and characterization of yeasts isolated from artisanal Fiore Sardo cheese. *International Journal of Food Microbiology, 95*(1), 51–59. https://doi.org/10.1016/j.ijfoodmicro.2004.02.001

Fairbairn, S., McKinnon, A., Musarurwa, H. T., Ferreira, A. C., & Bauer, F. F. (2017). The Impact of Single Amino Acids on Growth and Volatile Aroma Production by Saccharomyces cerevisiae Strains. *Frontiers in Microbiology, 8*, 2554. https://doi.org/10.3389/fmicb.2017.02554

Ghabbour, N., Lamzira, Z., Thonart, P., Cidalia, P., Markaoui, M., & Asehraou, A. (2011). Selection of oleuropein-degrading lactic acid bacteria strains isolated from fermenting Moroccan green olives. *Grasas y Aceites, 62*. https://doi.org/10.3989/gya.055510

Golomb, B. L., Morales, V., Jung, A., Yau, B., Boundy-Mills, K. L., & Marco, M. L. (2013). Effects of pectinolytic yeast on the microbial composition and spoilage of olive fermentations. *Food Microbiology, 33*(1), 97–106. https://doi.org/10.1016/j.fm.2012.09.004

Gonzalez-Garcia, R. A., McCubbin, T., Navone, L., Stowers, C., Nielsen, L. K., & Marcellin, E. (2017). Microbial Propionic Acid Production. *Fermentation, 3*(2), 21. https://doi.org/10.3390/fermentation3020021

Guinebretiere, M.-H., & Nguyen-The, C. (2003). Sources of Bacillus cereus contamination in a pasteurized zucchini purée processing line, differentiated by two PCR-based methods. *FEMS Microbiology Ecology, 43*(2), 207–215. https://doi.org/10.1111/j.1574-6941.2003.tb01060.x

Guinet, R. M. (1985). Miniaturized durham tubes for carbohydrate fermentation tests by yeasts. *Mykosen, 28*(8), 394–399. https://doi.org/10.1111/j.1439-0507.1985.tb02150.x

Harrell & Dupont (2020). F.E.J. Harrell, C. Dupont Hmisc: Harrell Miscellaneous. R Package Version 4.4-0 Available from https://CRAN.R-project.org/package=Hmisc.

Hernández, A., Martín, A., Aranda, E., Pérez-Nevado, F., & Córdoba, M. G. (2007). Identification and characterization of yeast isolated from the elaboration of seasoned green table olives. *Food Microbiology, 24*(4), 346–351. https://doi.org/10.1016/j.fm.2006.07.022

Hernández, A., Martín, A., Córdoba, M., Benito, M., Aranda, E., & Pérez-Nevado, F. (2008). Determination of killer activity in yeasts isolated from the elaboration of seasoned green table olives. *International Journal of Food Microbiology, 121*, 178–188. https://doi.org/10.1016/j.ijfoodmicro.2007.11.044

Huang, C.-W., Walker, M. E., Fedrizzi, B., Gardner, R. C., & Jiranek, V. (2017). Hydrogen sulfide and its roles in Saccharomyces cerevisiae in a winemaking context. *FEMS Yeast Research, 17*(6). https://doi.org/10.1093/femsyr/fox058

International Olive Council (2019). Economic Affairs and Promotion Unit "World Table Olives Exports & Imports" International Olive Council Website. Available online at: https://www.internationaloliveoil.org/wp-content/uploads/2020/04/HO-W901... (accessed November 2021).

Johnson, R. L., & Mitchell, A. E. (2018). Reducing Phenolics Related to Bitterness in Table Olives [Review Article]. *Journal of Food Quality; Hindawi.*. https://doi.org/10.1155/2018/3193185

Kazou, M., Tzamourani, A., Panagou, E. Z., & Tsakalidou, E. (2020). Unraveling the Microbiota of Natural Black cv. Kalamata Fermented Olives through 16S and ITS Metataxonomic Analysis. *Microorganisms, 8*(5), 672. https://doi.org/10.3390/microorganisms8050672

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research, 41*(1), Article e1. https://doi.org/10.1093/nar/gks808

Kurtzman, C. P. (2011). Chapter 57—Pichia E.C. Hansen (1904). In C. P. Kurtzman, J. W. Fell, & T. Boekhout (Éds.), *The Yeasts (Fifth Edition)* (p. 685-707). Elsevier. https://doi.org/10.1016/B978-0-444-52149-1.00057-4.

Kurtzman, C. P., & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek, 73*(4), 331–371. https://doi.org/10.1023/A:1001761008817

Lanza, B. (2013). Abnormal fermentations in table-olive processing : Microbial origin and sensory evaluation. *Frontiers in Microbiology, 4*. https://doi.org/10.3389/fmicb.2013.00091

Lê, S., Josse, J., & Husson, F. (2008). FactoMineR : An R Package for Multivariate Analysis. *Journal of Statistical Software, 25*(1), 1–18. https://doi.org/10.18637/jss.v025.i01

Llorente, P., Marquina, D., Santos, A., Peinado, J. M., & Spencer-Martins, I. (1997). Effect of salt on the killer phenotype of yeasts from olive brines. *Applied and Environmental Microbiology, 63*(3), 1165–1167.

Mannazzu, I., Domizio, P., Carboni, G., Zara, S., Zara, G., Comitini, F., Budroni, M., & Ciani, M. (2019). Yeast killer toxins : From ecological significance to application. *Critical Reviews in Biotechnology, 39*(5), 603–617. https://doi.org/10.1080/07388551.2019.1601679

Marquina, D., Peres, C., Caldas, F. V., Marques, J. F., Peinado, J. M., & Spencer-Martins, I. (1992). Characterization of the yeast population in olive brines. *Letters in Applied Microbiology, 14*(6), 279–283. https://doi.org/10.1111/j.1472-765X.1992.tb00705.x

McMurdie, P. J., & Holmes, S. (2013). phyloseq : An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE, 8*(4), Article e61217. https://doi.org/10.1371/journal.pone.0061217

Medina, E., Ruiz-Bellido, M. A., Romero-Gil, V., Rodríguez-Gómez, F., Montes-Borrego, M., Landa, B. B., & Arroyo-López, F. N. (2016). Assessment of the bacterial community in directly brined Aloreña de Málaga table olive fermentations by metagenetic analysis. *International Journal of Food Microbiology, 236*, 47–55. https://doi.org/10.1016/j.ijfoodmicro.2016.07.014

Montaño, A., Cortés-Delgado, A., Sánchez, A. H., & Ruiz-Barba, J. L. (2021). Production of volatile compounds by wild-type yeasts in a natural olive-derived culture medium. *Food Microbiology, 98*, Article 103788. https://doi.org/10.1016/j.fm.2021.103788

Panagou, E. Z., & Tassou, C. C. (2006). Changes in volatile compounds and related biochemical profile during controlled fermentation of cv. Conservolea green olives. *Food Microbiology, 23*(8), 738–746. https://doi.org/10.1016/j.fm.2006.02.005

Penland, M., Deutsch, S.-M., Falentin, H., Pawtowski, A., Poirier, E., Visenti, G., Le Meur, C., Maillard, M.-B., Thierry, A., Mounier, J., & Coton, M. (2020). Deciphering Microbial Community Dynamics and Biochemical Changes During Nyons Black Olive Natural Fermentations. *Frontiers in Microbiology, 11*. https://doi.org/10.3389/fmicb.2020.586614

Penland, M., Mounier, J., Pawtowski, A., Tréguer, S., Deutsch, S.-M., & Coton, M. (2021). Use of metabarcoding and source tracking to identify desirable or spoilage autochthonous microorganism sources during black olive fermentations. *Food Research International, 144*, Article 110344. https://doi.org/10.1016/j.foodres.2021.110344

Pereira, E. L., Ramalhosa, E., Borges, A., Pereira, J. A., & Baptista, P. (2015). YEAST dynamics during the natural fermentation process of table olives (Negrinha de Freixo cv.). *Food Microbiology, 46*, 582–586. https://doi.org/10.1016/j.fm.2014.10.003

Peres, C. M., Peres, C., & Xavier Malcata, F. (2017). Chapter 22—Role of Natural Fermented Olives in Health and Disease. In I. J. Frias, C. Martinez-Villaluenga, & E. Peñas (Eds.), *Fermented Foods in Health and Disease Prevention* (pp. 517–542). Academic Press. https://doi.org/10.1016/B978-0-12-802309-9.00022-4.

Pino, A., De Angelis, M., Todaro, A., Van Hoorde, K., Randazzo, C. L., & Caggia, C. (2018). Fermentation of Nocellara Etnea Table Olives by Functional Starter Cultures at Different Low Salt Concentrations. *Frontiers in Microbiology, 9*. https://doi.org/10.3389/fmicb.2018.01125

Panagou, E. Z., Schillinger, U., Franz, C. M. A. P., & Nychas, G.-J. E. (2008). Microbiological and biochemical profile of cv. Conservolea naturally black olives during controlled fermentation with selected strains of lactic acid bacteria. *Food Microbiology*, 25(2), 348-358. https://doi.org/10.1016/j.fm.2007.10.005.

Psani, M., & Kotzekidou, P. (2006). Technological characteristics of yeast strains and their potential as starter adjuncts in Greek-style black olive fermentation. *World Journal of Microbiology and Biotechnology, 22*(12), 1329–1336. https://doi.org/10.1007/s11274-006-9180-y

Ramírez, E., Brenes, M., García, P., Medina, E., & Romero, C. (2016). Oleuropein hydrolysis in natural green olives : Importance of the endogenous enzymes. *Food Chemistry, 206*, 204–209. https://doi.org/10.1016/j.foodchem.2016.03.061

Randazzo, C. L., Todaro, A., Pino, A., Pitino, I., Corona, O., & Caggia, C. (2017). Microbiota and metabolome during controlled and spontaneous fermentation of Nocellara Etnea table olives. *Food Microbiology, 65*, 136–148. https://doi.org/10.1016/j.fm.2017.01.022

Romeo, F. V. (2012). Microbiological Aspects of Table Olives. *Olive Germplasm - The Olive Cultivation, Table Olive and Olive Oil Industry in Italy.*. https://doi.org/10.5772/51479

Tassou, C. C., Panagou, E. Z., & Katsaboxakis, K. Z. (2002). Microbiological and physicochemical changes of naturally black olives fermented at different temperatures and NaCl levels in the brines. *Food Microbiology, 19*(6), 605–615. https://doi.org/10.1006/fmic.2002.0480

Tofalo, R., Perpetuini, G., Schirone, M., Suzzi, G., & Corsetti, A. (2013). Yeast biota associated to naturally fermented table olives from different Italian cultivars. *International Journal of Food Microbiology, 161*(3), 203–208. https://doi.org/10.1016/j.ijfoodmicro.2012.12.011

Toju, H., Tanabe, A. S., Yamamoto, S., & Sato, H. (2012). High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. *PLOS ONE, 7*(7), Article e40863. https://doi.org/10.1371/journal.pone.0040863

Vaughn, R. H., Stevenson, K. E., Davé, B. A., & Park, H. C. (1972). Fermenting Yeasts Associated with Softening and Gas-Pocket Formation in Olives. *Applied Microbiology, 23*(2), 316–320.

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis* ((2e éd.).). Springer International Publishing.

Woods, D. R., & Bevan, E. A. (1968). Studies on the Nature of the Killer Factor Produced by Saccharomyces cerevisiae. *Microbiology, 51*(1), 115–126. https://doi.org/10.1099/00221287-51-1-115

Zinno, P., Guantario, B., Perozzi, G., Pastore, G., & Devirgiliis, C. (2017). Impact of NaCl reduction on lactic acid bacteria during fermentation of Nocellara del Belice table olives. *Food Microbiology, 63*, 239–247. https://doi.org/10.1016/j.fm.2016.12.001

Zorić, N., Kopjar, N., Bobnjarić, I., Horvat, I., Tomić, S., & Kosalec, I. (2016). Antifungal Activity of Oleuropein against Candida albicans—The In Vitro Study. *Molecules, 21*(12), 1631. https://doi.org/10.3390/molecules21121631