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Use of metabarcoding and source tracking to identify desirable or spoilage autochthonous microorganism sources during black olive fermentations

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ABSTRACT

This study aimed at investigating the influence of the process environment and raw materials as sources of microorganisms during Nyons black table olive fermentations. Fermented olives and/or brine from spoiled fermentation tanks were analyzed and compared to good quality samples from fermentations collected during 3 consecutive harvest years. Fresh olives, salt and different process environment samples were also analyzed. Microbial diversity of all samples was analyzed using 16S and ITS2 amplicon sequencing and SourceTracker tool was used to investigate links between environment, raw materials and fermentation samples. First, comparison of microbial diversity in control and most spoiled fermentations revealed striking differences in bacterial composition with an overall higher diversity in spoiled fermentations especially for lactic acid bacteria with *Lentilactobacillus buchneri, Lentilactobacillus parafarraginis* dominating in brine and *Pediococcus parvulus, Pediococcus ethanolidurans* dominating in olive fruits. Fungal communities were similar in composition although higher abundances of *Pichia membranifaciens* and *Penicillium carneum/roqueforti* were observed in spoiled samples. Secondly, process environment samples were characterized by high bacterial and fungal diversity, especially compared to fresh olive fruits. Overall, dominant fungal species in control fermentations were also found in most environmental samples revealing a "house mycobiota". SourceTracker analysis further highlighted the contribution of brine and water from the optical sorter as a source of fungi. Most interestingly, spoilage fungi and most bacteria were retrieved in brine and environmental samples while others such as *P. ethanolidurans* were only found in environmental samples indicating that the studied spoilage originated from a fermentation deviation rather than a punctual contamination. Taken altogether, these results highlighted the positive and negative influence of the process environment and emphasized the relevance of studying it to better understand microbial vectors occurring during food fermentations, especially natural ones.

1. Introduction

Table olives are among the most consumed fermented vegetables worldwide with nearly 2.6 million tons produced in 2019 (International Olive Council, 2019). Although native to the Mediterranean region, they are produced and consumed worldwide. Table olives are highly appreciated by consumers for their pleasant tastes and aromas as well as for their health benefits (Conte et al., 2020). Table olives can be prepared using three main processes described as either the Spanish-style process (lye treated green olives prior to fermentation in brine), Californian-style preparation (ripe olives chemically oxidized) or Greek-style process (naturally fermented black olives in brine) (Romeo, 2012).

Nyons table olives are black table olives with PDO status produced in southern France. They are prepared using the Greek-style process and according to PDO specifications: Tanche variety olives, harvested at full maturity during the winter season, are directly submerged in 10% salt brine and slowly fermented for up to 1.5 years. Nyons table olives microbiota was recently explored (Penland et al., 2020) and as often observed in many spontaneous or natural fermentations, harbored a very rich and complex microbiota. A core mycobiota, composed of *Wickerhamomyces anomalus, Citeromyces nyonsensis, Zygotorulaspora mrakii, Pichia membranifaciens* and *Candida boidinii,* was determined while the bacterial microbiota was less complex and dominated by *Celerinatantimonas diazotrophica* throughout all the studied

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fermentations.

Although the complex microbial diversity of naturally fermented table olives is often related to complex organoleptic characteristics, it can also harbour unwanted microorganisms which may compromise both product safety and quality. Bacteria-associated defects in fermented olives are most frequently linked to off-flavours and off-odors (Lanza, 2013) while fungal defects are often associated with visual defects such as cloudy brines or gas pocket formations under the olive skin, especially when fermentative yeast abundances are too high (Arroyo-López et al., 2012). So far, three main sensorial spoilage phenomena have been described in table olives: "Zapateria", putrid, and butyric defects which are linked to *Propionibacterium*, *Desulfovibrio* and *Clostridium* genera metabolisms, respectively.

Process environment has also been shown to be a major reservoir of microorganisms involved in the fermentation process of many foods. This has been particularly explored in cheese. It has now been well established that vats, milking materials and ripening trays all harbour microbial biofilms that can enrich the cheese microbiota (Calasso et al., 2016; Montel et al., 2014). In line with this, Bokulich & Mills (2013) also showed that dairy-plant surfaces contributed to the cheese microbiota. In addition, artisanal and/or spontaneous fermentation processes imply higher exposition to contaminants through the process environment compared to automated industrial ones. Investigating the links between the microbial communities encountered during fermentation and the in-house microbiota is clearly of interest to better manage and control fermentation processes. Despite this fact, and to our best knowledge, no study has yet investigated the influence of the processing environment on table olive fermentations.

Recent studies on microbial communities in many foods have greatly benefited from state-of-the-art DNA-based and Next Generation Sequencing (NGS) techniques (De Filippis et al., 2018; Frigerio et al., 2020). Indeed, they provide in-depth and accurate data on microbial composition in a given sample and at a given time during fermentation without the fastidious need to cultivate microorganisms on a wide range of media and identify massive numbers of individual isolates (Galimberti et al., 2020; Pasolli et al., 2020). Thus, these techniques are used more and more frequently to compare food communities, monitor microbial changes during fermentation but also investigate potential microbial vectors. SourceTracker (Knights et al., 2011) is a relatively recent bioinformatic tool based on Bayesian approach that aims at predicting the source of microbial communities in designated sink samples. It actually traces and proposes sources of microorganisms.

In this context, our study aimed at (i) identifying microbial species linked to spoiled Nyons table olives by comparing spoiled and control fermentation samples and (ii) investigating the impact of raw materials and process environment on these samples using high-throughput

Table 1
Description of the samples used in this study.

	Sample code	Harvest period	Description (Tank – sample type and age -spoilage profile)
Control fermentations	Normal fermentations with final product characteristics complying with PDO and producer requirements		
	C1_D64	2018	C1 - Olive fruits (O) and brine (B) at day 64
	C2_D64	2018	C2 - Olive fruits (O) and brine (B) at day 64
	C1_D183	2018	C1 - Olive fruits (O) and brine (B) at day 183
	C2_D183	2018	C2 - Olive fruits (O) and brine (B) at day 183
	C1_End	2018	C1 - Olive fruits (O) and brine (B) at the end of fermentation
	C2_End	2018	C2 - Olive fruits (O) and brine (B) at the end of fermentation
	C2017_End	2017	C2017 - Brine sample at the end of fermentation
	C2016_End	2016	C2016 - Brine sample at the end of fermentation
Spoiled fermentations	Olive fermentation tanks identified as spoiled by the producer based on olfactive and visual criteria		
	S1_End	2016	S1 - Brine (B) and olives fruit (O) at the end of fermentation - Profile 1*
	S2_End	2016	S2 - Brine (B) and olives fruit (O) at the end of fermentation - Profile 1*
	S3_End	2016	S3 - Brine (B) and olives fruit (O) at the end of fermentation - Profile 1*
	S4_End	2016	S4 - Brine (B) and olives fruit (O) at the end of fermentation - Profile 1*
	S5_End	2016	S5 - Brine (B) and olives fruit (O) at the end of fermentation - Profile 1*
	S6_End	2016	S6 - Brine (B) and olives fruit (O) at the end of fermentation - Profile 1*
	S7_End	2016	S7 - Brine (B) and olives fruit (O) at the end of fermentation - Profile 1*
	S2016_End	2016	Tank S2016 – Brine at the end of fermentation - Profile 2**
Raw materials			
	Salt_A	2018	Organic dry sea salt (1/10 diluted)
	Salt_B	2018	Conventional dry sea salt (1/10 diluted)
	FF_1	2018	Tank C1 - Fresh olives fruits
	FF_2	2018	Tank C2 - Fresh olives fruits
	FB_1	2018	Tank C1 - Fresh brine (10% salt)
	FB_2	2018	Tank C2 - Fresh brine (10% salt)
Process environment			
	Optical_sorter_surf	2018	Optical sorter surface
	PF_treadmill_surf	2018	Post-fermentation sorting treadmill
	Wall_surf	2018	Wall surface
	DrainingS_A	2018	Draining sytem grill A
	DrainingS_B	2018	Draining sytem grill B
	Cond_treamill	2018	Conditionning sorting treadmill
	Filtered_brine	2018	Filtered brine used for olive packaging
	St_water_A	2018	Stagnant water in storage area A
	St_water_B	2018	Stagnant water in storage area B
	Optical_water	2018	Water on optical sorter
	DS_water	2018	Water from draining system in tank preparation area
	Washing-water	2018	Washing water

* Spoilage profile 1 - Strong acetic smell, friable olives and cloudy, thick brine.
** Spoilage profile 2 – Olives with brown spots and small gas pockets on the surface.

screening (HTS) metagenetic analyses and SourceTracker tool.

2. Material & methods

2.1. Olive fermentation process and sampling campaign

All samples were obtained from the same producer and were collected from the same facilities. Fermentation tanks were prepared with the same traditional process described by Nyons table olives PDO requirements and treated in the same conditions: fresh olive fruits were washed in water, sorted on treadmills before being put in tanks (300 kg) and submerged in 10% salt brines (200 L). Tanks were then closed with a heavy lid for the remaining of the fermentation and stored in the same area at a temperature below 18 °C.

Samples consisted of olives and/or brines from spoiled fermentation tanks and normal ones (used as controls) as well as raw materials and process environment samples. The samples were collected over three different harvest periods from 2016 to 2018. Details regarding the choice and description of these samples are provided below and summarized in Table 1.

2.1.1. Spoiled Nyons table olive fermentation samples

Spoiled samples corresponded to eight different olive tanks from the 2016 harvest period. Samples S1_End to S7_End were singled out at the end of fermentation among the other tanks from the 2016 harvest period based on their abnormal profile. They were all characterized by a strong acetic and overripe-fruit smell. Brines were thicker and cloudier, while olive fruits were more friable or brittle (when cut) than control fruits. No visual defects such as discoloration or gas pockets were noticeable on fruits. S2016_End sample was characterized by the presence of brownish spots and gas pocket on the surface of the olive fruit skin but no olfactive defect was noticeable.

2.1.2. Control Nyons table olive fermentation samples

Control samples were collected from three different harvest period fermentations: 2016 (C2016), 2017 (C2017) and 2018 (C1 & C2 tanks). All tanks were sampled and analyzed at the end of fermentation. C1 and C2 tanks corresponded to both olive and brine samples from previously studied Nyons olive fermentations as reported by Penland et al. (2020). These tanks were included as controls since they were fully characterized and the fermentation outcomes were consistent with PDO requirements. In addition, samples collected at 3 intermediary stages of C1 and C2 fermentations (corresponding to raw materials at day 1, day 64 and day 183 of fermentation) were analyzed in order to reflect microbial diversity and dynamics at other fermentation stages.

2.1.3. Process environment and raw materials samples

Sampling was performed at different places in the production and storage facilities to track potential microbial sources. These samples were collected at the start (day 8) of the 2018 harvest fermentations (control fermentations) from different zones in the Nyons olive production facility. Four surfaces were also analyzed by streaking swabs moistened with Tryptone Salt diluent (TS) over a 71 cm^2 area in different working zones. Finally, two dry sea salt samples, used as raw materials for brine preparation, were taken. All samples were analyzed according to the protocol described below and within 24 h.

2.2. pH and microbial monitoring by culture-dependent approach

2.2.1. Fermentation brine pH measurement

pH values were measured for each fermentation sample using a pH meter (Hanna Instruments HI 2020–02).

2.2.2. Enumeration of microbial populations in spoiled and control fermentations or environmental samples

Microbial populations were monitored for both control and spoilage samples. Brine and olive fruits were treated together in a 1:1 ratio (w/w): 12.5 g of olive flesh and 12.5 mL of brine were mixed with 225 mL Buffered Peptone Water and blended with a stomacher for 3 min at high speed. Serial dilutions were then prepared in Tryptone Salt diluent (TS; sodium chloride 8.5 g/L, tryptone 1 g/L) and plated on seven different media to enumerate total fungal populations (Yeast extract Glucose Chloramphenicol medium, YGC, 25 °C, 5 days), halotolerant fungi (YGC + 5% NaCl, 25 °C, 5 days), total 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 addition, hygiene and safety quality of both control and spoiled olives was assessed in compliance with EC Regulation n°2073/2005 (Commission Regulation (EC) No 2073/2005 of 15 November 2005 on Microbiological Criteria for Foodstuffs, 2005). *Escherichia coli* (TBX, 42 °C, 24 h) and coagulase-positive staphylococci (Baird Parker supplemented with Rabbit Plasma Fibrinogen, 37 °C, 48 h) were enumerated while *Listeria monocytogenes* and *Salmonella* spp. absence in 25 g was verified, following ISO 11290-1:2017 and ISO 6579-1:2017 guidelines, respectively.

Regarding environmental samples, mesophilic aerobic microorganisms were enumerated on PCA (30 °C, 72 h) and fungi on YGC (25 °C; 5 days) after initial serial dilutions in Tryptone-Salt diluent.

2.3. Microbial community analyses using metagenetics

2.3.1. DNA extraction

DNA was extracted from both brine and olive fruit samples for control and spoiled fermentations. Cell pellets were prepared as described in Penland et al. (2020). Regarding environmental samples, pellets were obtained from liquid samples by centrifuging 1 mL (9000*g*, 15 min, 4 °C), whereas for surface samples, swabs were placed in 1 mL of TS diluent prior to centrifugation (9000*g*, 15 min, 4 °C) to obtain cell pellets. All DNA extractions were performed using NucleoSpin Soil DNA kit (Macherey-Nagel, Germany) following the protocol previously described in Penland et al. (2020). DNA extracts were stored at −20 °C.

2.3.2. Amplification and sequencing

DNA extracts were used to study bacterial and fungal diversity in all samples. DNA extracts were first submitted to PCR amplification. For bacteria, V3-V4 region of the 16S rDNA 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, ITS3f/ITS4_Kyo1 primers targeting ITS2 region and PCR conditions as described by Toju et al. (2012) were used. Sequencing was performed at GATC sequencing platform (Eurofins, Germany). All ITS2 and V3-V4 amplicons were sequenced in the same run using Illumina Miseq PE300 technology generating 2x300 bp reads.

2.3.3. Bioinformatics

Sequencing data were then analyzed using the FROGS pipelines developed by Escudié et al. (2018) under Galaxy (Afgan et al., 2018). Briefly, raw paired-end reads were first assembled and filtered based on the following criteria: only sequences with amplicon size between 370 and 490 bp for V3-V4 contigs and 100–530 bp for ITS2 contigs were kept while the ones with a mismatch rate above 0.1 and the sequences with NNN were filtered out for both data types. Then, for each data type, remaining sequences were respectively clustered using the Swarm algorithm (Mahé et al., 2015) with an aggregation distance of 3 into Operational Taxonomic Units (OTUs). Sequences underwent some filtering steps: chimeras were detected and removed using Uparse (Edgar, 2013) using 'de novo parameter', then sequences with a relative abundance below 5×10^{-5} or present in only one sample were excluded. For ITS2 data, ITSx was used prior to affiliation to extract and select ITS2

sequences (Bengtsson-Palme et al., 2013). Finally, affiliation step was performed using SILVA (V138) and UNITE 8.2 fungi databases for 16S and ITS2 data, respectively. When species identification by blastn + and identification was below 97%, affiliation was manually corrected to the genus level. In the same manner, when sequences were multi-affiliated by FROGS, because the targeted V3-V4 region or ITS2 were unable to discriminate species, the resulting species or genus-level assignations were implemented into the final OTU table. OTUs affiliated to chloroplasts or mitochondrial sequences in the 16S data set were excluded from the analyses.

2.4. Metagenetic statistical data analyses

Processing and statistical analyses of microbial communities were performed using Phyloseq package (McMurdie & Holmes, 2013) under R software. Alpha and beta-diversity analyses were performed on control and spoiled fermentation sample datasets. Data were first normalized based on the sample that had the lowest number of sequences. Taxonomic composition and abundance distribution were then determined and alpha-diversity indexes (Observed, Chao1 and Shannon) calculated. Kruskal-Wallis test was used to compare values between spoiled and control fermentation samples. When differences were significant ($P < 0.05$), the Wilcoxon test was performed to explore differences within the variable. Weighted UniFrac distance and Bray-Curtis distance were calculated to analyze beta-diversity within fungal and bacterial communities. Multidimensional Scaling (MDS) and Adonis test (999 permutations) in R vegan package (Oksanen et al., 2013), were then used to assess the impact of the same variables than for alpha-diversity. Moreover, OTU abundance difference analysis between control and spoiled samples was performed on the non-rarefied OTU count table using Deseq2 package (Love et al., 2014; McMurdie & Holmes, 2014; Safari et al., 2020). Differences were expressed as log2 fold changes and their significance levels were tested using Wald test followed by Benjamini-Hochberg False Discovery Rate correction with alpha value set at 0.05 (Benjamini & Hochberg, 1995). Statistical tests (ANOVA, Kruskal-Wallis, Wilcoxon and Adonis test) were performed under R software using FactoMiner (Lê et al., 2008) and Hmisc (Hmisc: Harrell Miscellaneous. R package version 4.5–0, 2021) packages and XLSTAT software (Addinsoft, Paris, France). Representations were obtained using R ggplot2 package (Wickham, 2016).

2.5. Source tracker analyses

SourceTracker algorithm (Knights et al., 2011) was used to investigate possible sources of contamination (environmental sources and raw materials, i.e. salt, olive fruits, brine) in spoiled and unspoiled brines and olives during fermentation. The analysis was carried out on samples with more than 1000 reads at a rarefaction depth of 1159 reads for 16S rRNA and 1961 for ITS amplicons, with 100 burn-ins and 10 restarts. Venn diagrams were built from non-rarefied OTU tables using jvenn online tool (Bardou et al., 2014).

3. Results

3.1. Sequencing performance

Regarding ITS2 sequencing data, a total of 1 478 832 quality-filtered contigs (average length of 291 bp) were obtained through ITS2 Illumina sequencing. After chimera and singleton removal, sequences were clustered into 165 OTUs belonging to Ascomycota (94.2%), Basidiomycota (5.6%) while 0.2% were unidentified. After normalization, 1961 sequences per sample were kept for diversity analyses.

V3-V4 16S rDNA gene Illumina sequencing resulted in 1 556 969 reads which passed quality filtering with an average length of 427 bp. After assignation and contaminant removal, 524 958 sequences were left and clustered into 144 OTUs that were assigned at the genus or species level (96% sequences with a coverage at 100% and identity between 95 and 100%). However, sequence numbers highly differed between samples and were particularly low for environmental and raw material samples. For this reason, control and spoilage fermentation sample diversity analysis was conducted separately from environment samples. Alpha and beta-diversity analyses were performed after rarefaction at 1159 sequences per sample.

3.2. Microbiota of spoiled and control Nyons table olive fermentations

3.2.1. Microbial populations and pH determinations in control and spoiled fermentations

Safety and hygienic quality of both control and spoiled olive fermentation samples at the end of fermentations were tested according to EU regulation 2073/2005. All criteria were satisfied: no *Salmonella* or *Listeria* were detected in 25 g of sample, *Escherichia coli,* coagulase-positive staphylococci and enterobacteria populations were below detection level (<100 CFU/g) in all samples.

Based on the microbial populations enumerated and pH values determined for samples, major differences were observed between spoiled and control fermentations (Table 2). At the end of fermentation, pH values were significantly lower ($P < 0.001$) for spoiled samples, with values near ~ 3.8 versus ~ 4.4 in control samples. Moreover, lactic acid bacteria (LAB) and halotolerant LAB were at significantly higher counts in spoiled samples. Levels reached 4.5 to 5 log CFU/g in the analyzed brine/olive mixtures while they were undetected (<2.3 $\log_{10}$ CFU/g) during control fermentations ($P < 0.001$). No significant differences in fungal counts were observed. Populations ranged between 3.85 and 4.80 $\log_{10}$ CFU/g in spoiled samples and were close to 4.5 $\log_{10}$ CFU/g in control samples at the end of fermentation.

3.2.2. Comparison of fungal communities between spoiled and control fermentations

Alpha-diversity indexes (Observed and Chao1 for richness, Shannon for evenness) did not show any significant differences between spoiled and control fermentation samples regardless of the matrix type (brine or olive fruit). The only significant difference was observed for Shannon evenness index between olives and brines ($P < 0.05$) with higher values observed for olive fruits compared to brine samples (**Supplementary Fig. S1**).

To assess beta-diversity, MDS analysis based on weighted UniFrac distance was chosen as it explained the highest variance with nearly 71% on the first two dimensions compared to Bray Curtis distance (42.3%) (Fig. 1). Axis 1 (38.6%) clearly separated spoiled fermentation samples (no distinction was made between the type of sample: brine, dry salt, and olive samples) from control samples, while on axis 2 (32.3%), raw material and control fermentation samples were opposed. Interestingly, process environment samples were not homogeneously distributed with most of surface samples grouped close to control fermentations along axis 2. Adonis tests performed on the different variables (category and type of samples) confirmed these observations with significant differences ($P < 0.001$ and $R^2 = 0.47$) between communities from spoiled and control fermentations. Overall, alpha and beta-diversity suggested that fungal communities from spoiled fermentations differed more by the relative abundances of shared OTUs rather than the presence of OTUs specific to one community.

Further analyses were therefore performed to investigate differences in fungal community compositions between spoiled and control samples as well as according to matrix type (brine versus olive fruits). Based on the relative species abundances observed between samples (Fig. 2) and differences in species OTU abundances determined by Deseq analysis, similar conclusions could be made. Similarities in species composition were identified in all samples at the end of fermentation and *Pichia membranifaciens* dominated in all cases. However, control fermentations were also dominated by *Candida boidinii* and *Saccharomyces cerevisiae* while these species were subdominant in S1 to S7 spoiled fermentation

Table 2
Microbial population counts (log_{10} CFU/g of mixed olive and brine) and pH (Units) in spoiled and control fermentation samples. For control or spoiled samples, different letters in a row indicate a significant difference ($P < 0.05$) according to Tukey's LSD test.

Code	pH in brine	Mesophilic aerobic microorganisms	Halotolerant aerobic microorganisms	Anaerobic LAB*	Halotolerant anaerobic LAB*	Fungi	Halotolerant fungi	Enterobacteria
P value	**<0.0001**	**0.02**	0.08	**<0.0001**	**<0.0001**	**0.001**	**0.001**	
Control samples								
FF_1 & FB_1	7.53^{a}	2.79^{a}	2.79^{a}	$<DT^{a}$	<DT	3.01^{a}	2.3^{a}	<DT
FF_2 &FB_2	7.64^{a}	3.63^{a}	3.88^{a}	$<DT^{a}$	<DT	3.92^{a}	3.71^{a}	<DT
C1_D64	4.56^{b}	5.39^{b}	4.91^{a}	$<DT^{a}$	<DT	5.36^{d}	5.07^{b}	<DT
C2_D64	4.58^{b}	5.63^{b}	5.13^{a}	$<DT^{a}$	<DT	5.68^{d}	5.48^{b}	<DT
C1_D183	4.5^{b}	4.1^{b}	4.57^{a}	$<DT^{a}$	<DT	5.19^{dc}	5.04^{b}	<DT
C2_D183	4.54^{b}	5.29^{b}	5.25^{a}	$<DT^{a}$	<DT	5.56^{dc}	5.46^{b}	<DT
C1_End	4.49^{b}	4.69^{b}	4.44^{a}	$<DT^{a}$	<DT	4.42^{bc}	4.53^{b}	<DT
C2_End	4.43^{b}	4.86^{b}	4.67^{a}	$<DT^{a}$	<DT	4.87^{bc}	4.62^{b}	<DT
Spoiled samples								
S1_End	3.79^{c}	4.54^{b}	3.78^{a}	5.84^{b}	5.94^{b}	4.06^{b}	3.85^{b}	<DT
S2_End	3.83^{c}	5.19^{b}	4.95^{a}	6.06^{b}	5.92^{b}	4.91^{b}	4.78^{b}	<DT
S3_End	3.78^{c}	5.3^{b}	5.26^{a}	6.08^{b}	6.16^{b}	4.86^{b}	4.58^{b}	<DT
S4_End	3.78^{c}	5.11^{b}	4.96^{a}	6.05^{b}	6.10^{b}	4.48^{b}	4.34^{b}	<DT
S5_End	3.72^{c}	4.84^{b}	4.09^{a}	5.91^{b}	5.96^{b}	4.85^{b}	4.19^{b}	<DT
S6_End	3.85^{c}	5.49^{b}	5.33^{a}	5.93^{b}	5.97^{b}	4.31^{b}	4.39^{b}	<DT
S7_End	4.41^{c}	5.55^{b}	5.65^{a}	6.22^{b}	6.21^{b}	4.45^{b}	4.41^{b}	<DT

Abbreviations: LAB Lactic acid bacteria - DT Detection threshold (no colony on plate at the lowest dilution).
* ANOVA was performed with detection threshold value 2.3 log_{10} CFU/g corresponding to the highest possible concentration.

tanks. In these spoiled samples, higher abundances of some fungal species such as *Botrytis* sp. were also identified. Focusing on olive fruit communities, Deseq analyses were performed to determine OTU abundance differences and strong differences were observed for nine species with log2 fold change values ranging from 5 to 32 (Fig. 3). For instance, *Pichia membranifaciens* (log2 fold change |32|, $P < 0.001$), *Candida spencermartinsiae* (log2 fold change |24|, $P < 0.001$) and *Penicillium carneum/roqueforti* (log2 fold change |7|, $P < 0.001$) were at significantly higher abundances in spoiled fermentation samples as compared to control ones by the end of fermentation. Similar results were observed in brines for *P. membranifaciens* (log2 fold change |24|, $P < 0.001$) and *Penicillium carneum/roqueforti* (log2 fold change |24|, $P < 0.001$) although it was less pronounced in brines. In addition, other species (mostly filamentous fungi) were found at significantly higher abundances, i.e., *Neoascochyta graminicola* (log2 fold change |41|, $P < 0.001$), *Alternaria* sp. (log2 fold change |35|, $P < 0.001$), *Botrytis* sp. (log2 fold change |31|, $P < 0.001$) and *Epicoccum hordei/nigrum/proteae* (log2 fold change |39|, $P < 0.001$). However, for these latter species, it should be noted that although their relative abundances were significantly higher, most of them remained, overall, subdominant (<0.3% in average) in the spoiled brine and olive fruit fungal communities.

3.2.3. Bacterial community comparison between spoiled and control fermentations

Alpha-diversity analysis revealed significant differences within bacterial communities of spoiled and control fermentations (**Supplementary Fig. S2**). All three calculated indexes were significantly higher in spoiled olives and brines compared to their respective control counterparts ($P < 0.05$ for observed richness and $P < 0.01$ for Chao1 and Shannon indexes). Overall, the highest alpha-diversity values, all indexes considered, were observed for spoiled fermentation brines indicating that these samples were characterized by a more diverse and evenly distributed species community compared to control fermentations.

Beta-diversity was analyzed using MDS analysis on both Bray-Curtis and weighted UniFrac distances. MDS analysis based on Bray-Curtis distance explained the most variance on the first two axis (79.7%). It showed a clear separation of spoiled and control fermentation samples on axis 1 and further separated spoiled brines and olives on axis 2 (Fig. 4). Complementary Adonis test confirmed that the bacterial community structure in spoiled and control fermentations was significantly different ($P < 0.001$; $R^2 = 0.55$). However, differences between brines and olive fruits were not statistically significant and associated with a low R^2 value, therefore showing a lower impact of this variable on the bacterial communities ($P = 0.12$; $R^2 = 0.07$).

Differences in bacterial community profiles between control and spoiled samples were further explored by composition and OTU abundance difference analyses. Bacterial community composition at the species- and OTU-level confirmed trends observed through MDS analysis. Striking differences were observed with these two analyses. Communities from spoiled samples differed from control fermentations by both the species present and the dominant species abundances (Fig. 5) except for S2016_End sample. Control samples and surprisingly spoiled sample S2016_End showed similar profiles. Indeed, the bacterial community composition was stable during (at days 64, 183) and at the end of fermentation for all three considered harvest periods (C2016, C2017, C1 and C2 in 2018). For both olive fruits and brines, bacterial communities were largely dominated by *Celerinatantimonas diazotrophica* (relative abundances ranging from 85 to 100%) followed by *Marinobacterium litorale*.

Although both species were identified in S1 to S7 spoiled fermentation samples, they were sub-dominant and instead lactic acid bacteria (LAB) species dominated. In olive fruits, *Pediococcus parvulus* was clearly the most abundant LAB (30 to 85%), followed by *Lentilactobacillus buchneri* and *Lentilactobacillus parafarraginis*. Other LAB including *Pediococcus ethanolidurans, Lactiplantibacillus pentosus/plantarum* as well as

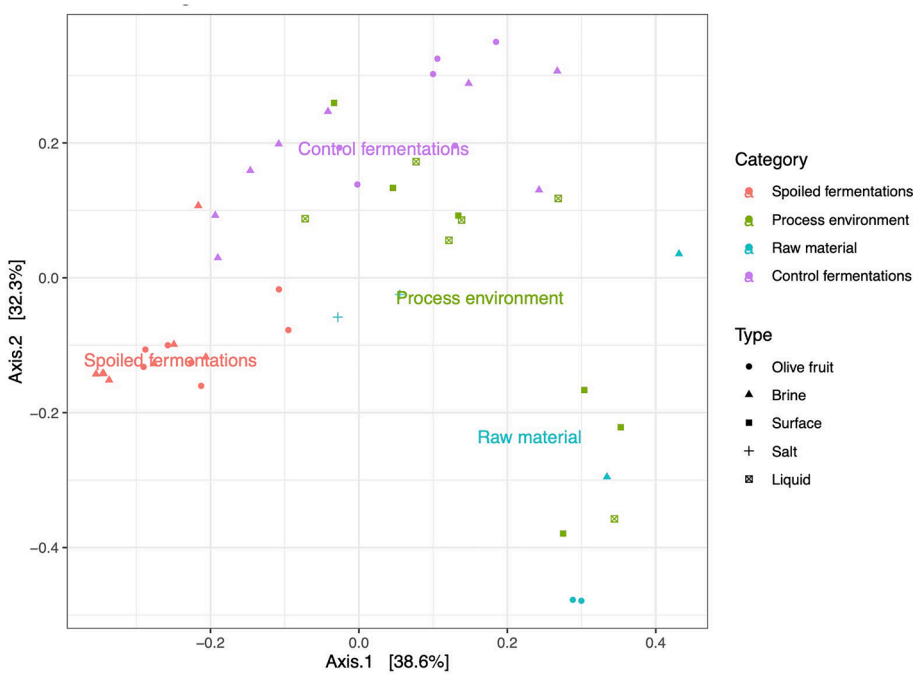

Fig. 1. Beta-diversity analysis of fungal communities by Multidimensional Scaling using weighted UniFrac distance. The four sample categories are considered: spoiled fermentations, control fermentations, process environment and raw materials and differentiated according to the type of samples.

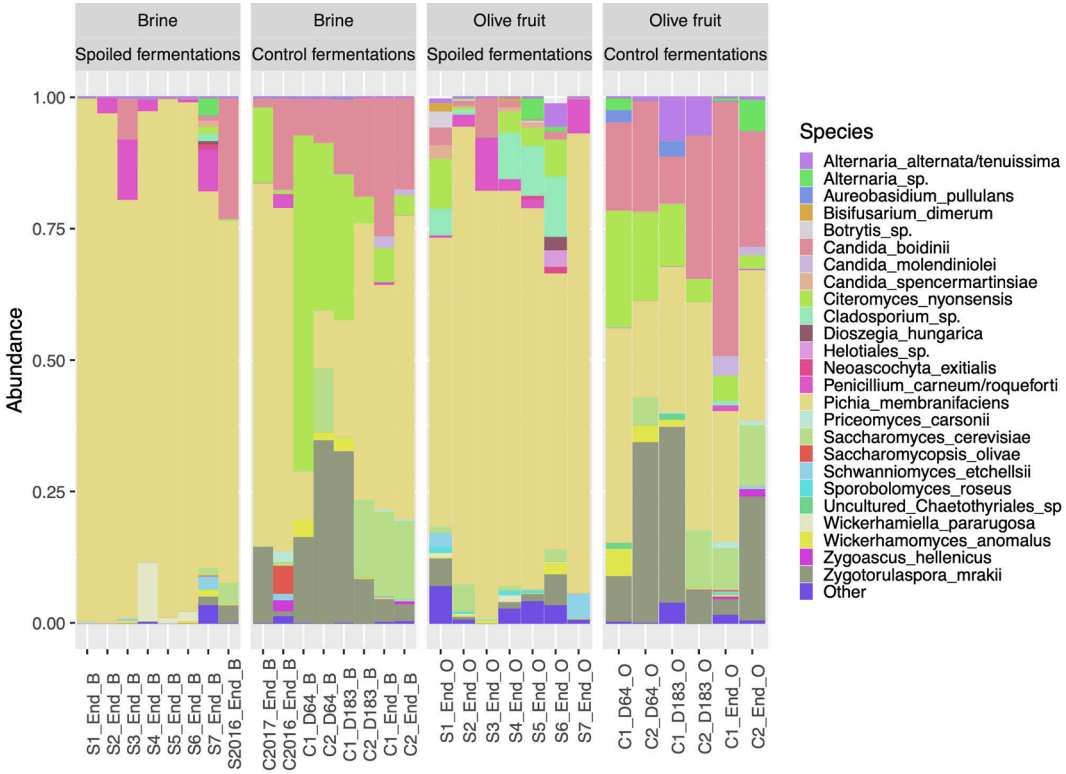

Fig. 2. Fungal community composition comparison of spoiled and control fermentation samples. Relative abundances of the 25 most abundant species are presented for brine and olive fruits separately, based on ITS2 region metagenetic analysis.

Suttonella indologenes (from the Proteobacteria phylum) were also found in most samples, but at lower abundances. In spoiled brines, bacterial communities were dominated by *L. parafarraginis* except for two samples. The previously identified species in olive fruit, namely *P. parvulus* and *L. buchneri,* were the second and third most dominant species. Overall, higher species diversity was observed in spoiled versus control samples.

Interestingly, for these seven samples, comparison of OTU abundances revealed significantly higher abundances for 79 and 31 OTUs (log2 change values between 8 and 31) in spoiled brine and olive fruit,

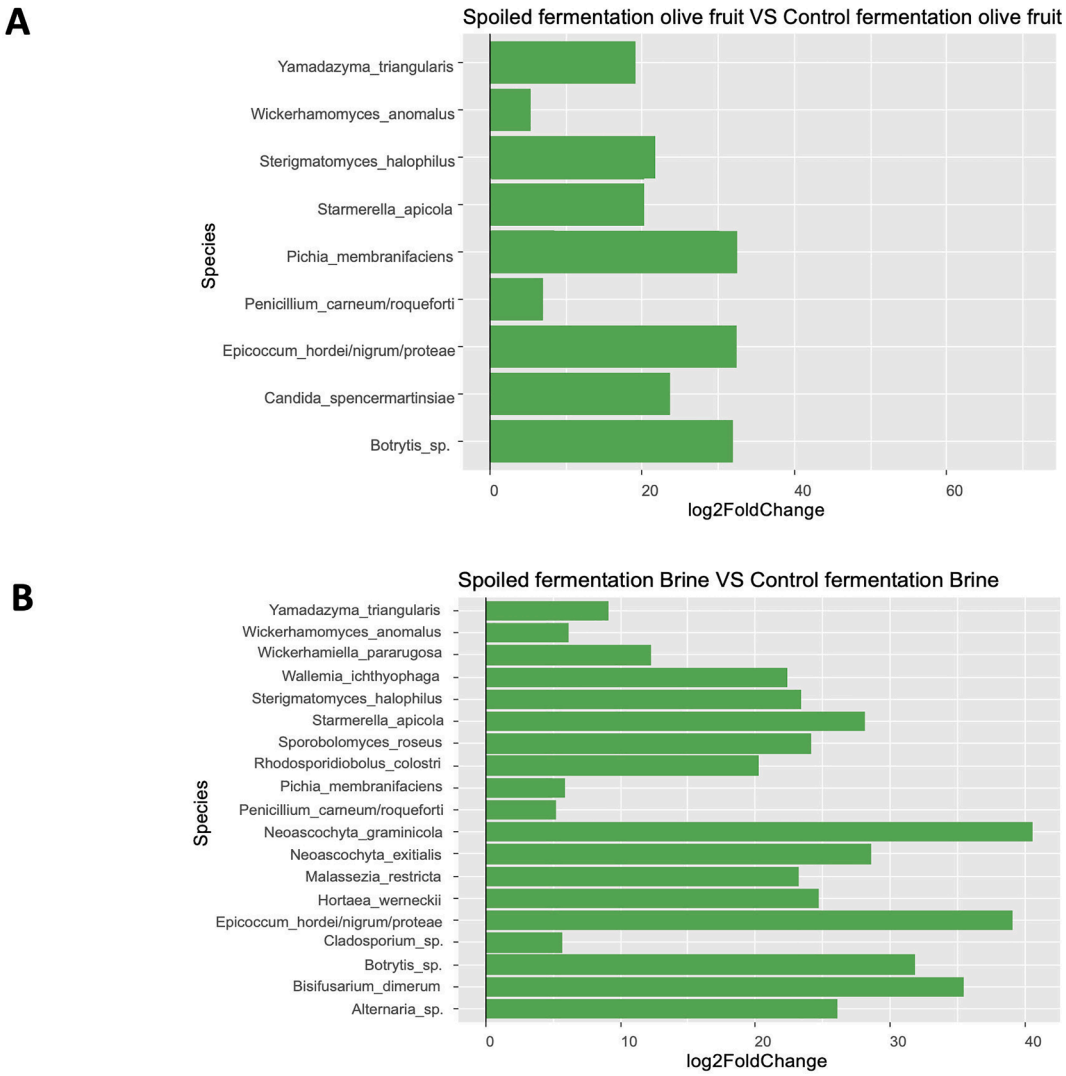

Fig. 3. OTUs differential abundances between spoiled and control fermentations (**A**) olive fruit and (**B**) brine. Log2 fold changes were calculated using Deseq2 tool. Only OTUs with a Pvalue < 0.05 (after adjustment by BH correction) are displayed.

respectively, when compared to control counterparts (data not shown). When looking at the OTU distribution for the different conditions, differences between control and spoiled samples were even more striking as most OTUs were completely absent in control fermentations (**Supplementary Fig. S3**). This included the dominant LAB species observed in spoiled fermentation samples as well as other species such as *Escherichia coli* and *S. indologenes*. Overall, bacterial communities in spoiled fermentations strongly differed in species composition and diversity, especially for LAB which were dominant in spoiled fermentations, and in the abundances of dominant species found in brines and olive fruits.

3.3. Tracking of microbial sources in process environment and raw materials

3.3.1. Fungal diversity analysis

Mycobiota in process environment samples and raw materials collected during control olive fermentations were analyzed by metagenetic analyses. The 25 most abundant species that were identified in the different samples are presented in Fig. 6**.** High fungal diversity was encountered in the process environment for both surface and liquid samples in comparison to raw materials. Highest OTU diversity was observed in liquid samples, such as the Optical_water sample (77 OTUs detected), which was also previously identified as a main contributor in fermentation samples. A similar trend was observed for samples belonging to the surface of the draining systems. Among raw materials, salt used to prepare brines showed the highest diversity.

Focusing on the distribution of fungal species, some of the most dominant throughout fermentation were *Pichia membranifaciens, Zygotorulaspora mrakii, Wickerhamomyces anomalus, Citeromyces nyonsensis* and *Candida boidinii.* They were widespread among environmental samples (i.e., liquids, surfaces) and brines for raw materials. Fungal communities encountered in process environment samples were mostly dominated by filamentous fungi. *Penicillium* and *Cladosporium* genera were particularly represented as well as some yeast species such as *Candida spencermartinsiae* and *Candida norvegica.* Both *Candida* species were present at high abundances in several surface and liquid process environment samples.

For raw materials, high diversity was observed in salt samples used for brines as more than 30 OTUs were identified. Highest species abundances mainly corresponded to the dominant yeasts encountered during fermentation. In comparison, fungal diversity on fresh olive fruits mainly consisted of *Alternaria alternata/tenuissima* and *Aureobasidium pullulans*. Neither species was found in high abundances in olives during fermentation.

Using the SourceTracker tool and ITS2 metagenetic data, nine

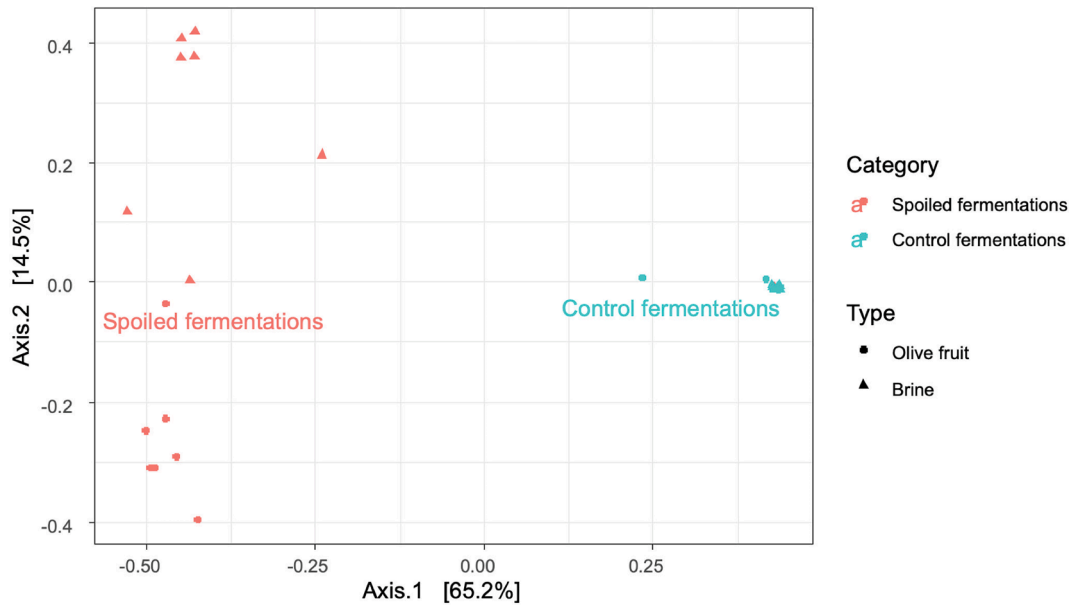

Fig. 4. Beta-diversity analysis of spoiled and control fermentation bacterial communities using Multidimensional Scaling on Bray-Curtis distance.

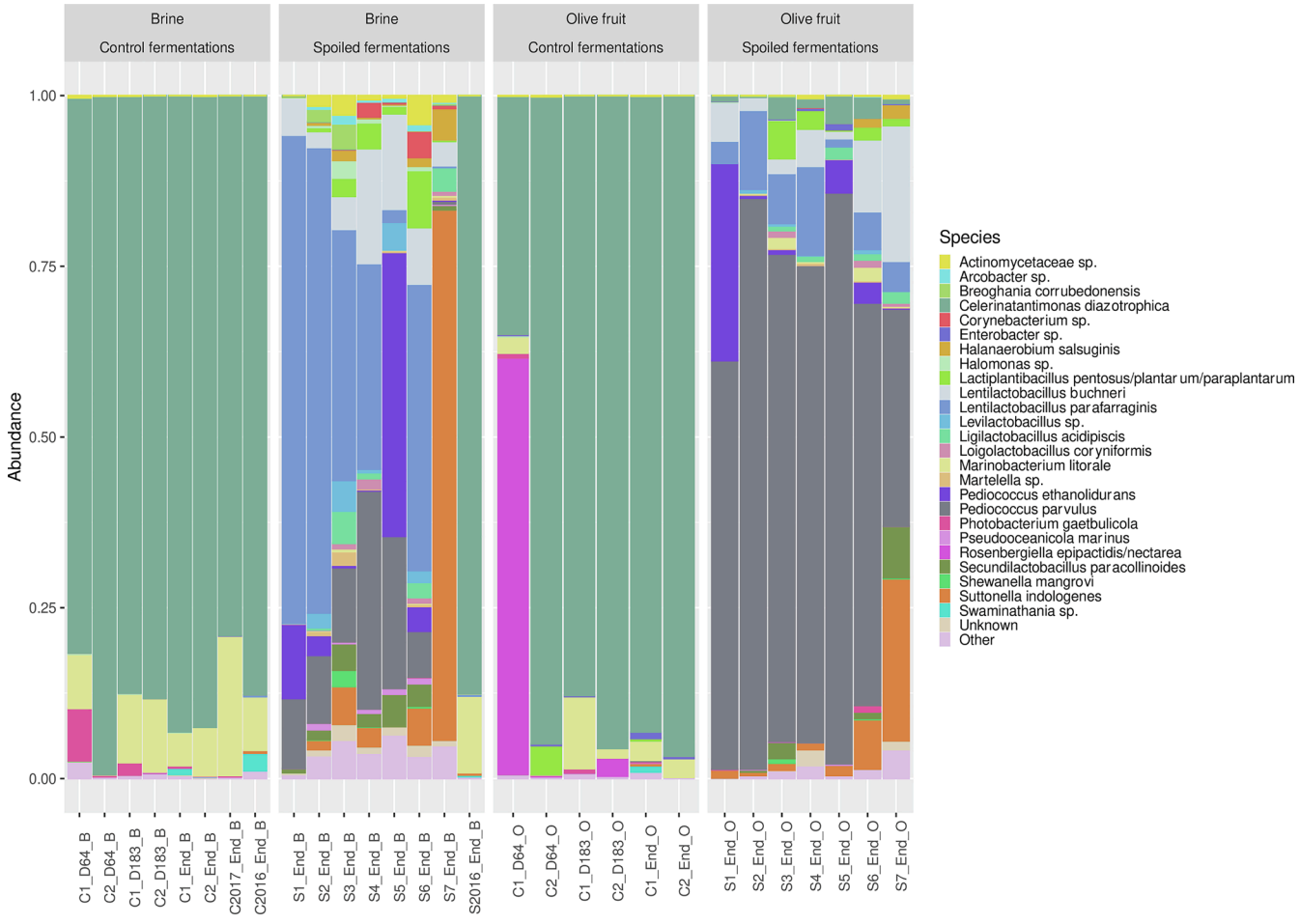

Fig. 5. Bacterial communities of spoiled and control Nyons olive fermentations as revealed by V3-V4 region of rRNA gene metabarcoding analysis. The 25 most abundant species are presented for control and spoiled fermentation samples with brine and olive fruit separated.

potential sources were analyzed and seven were shown to contribute to fungal community composition of at least one sample (Fig. 7). Moreover, the percentage of unknown sources ranged from 0.07% (C2017_Brine) to 15.4% (C2016_Brine). Overall, the salt used for brining was identified as the main contributor to fungal communities as observed in the different fermentation samples regardless of whether they were spoiled and the

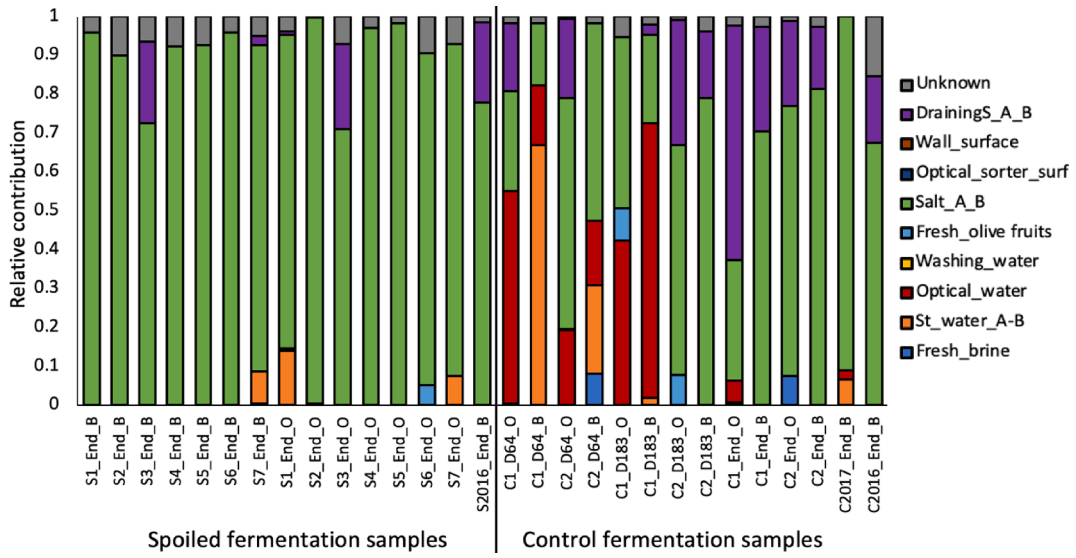

Fig. 6. Fungal diversity encountered in raw material and process environment samples in the olive fermentation-plant based on ITS2 metagenetic analysis. Relative abundances of the 25 most abundant species are presented.

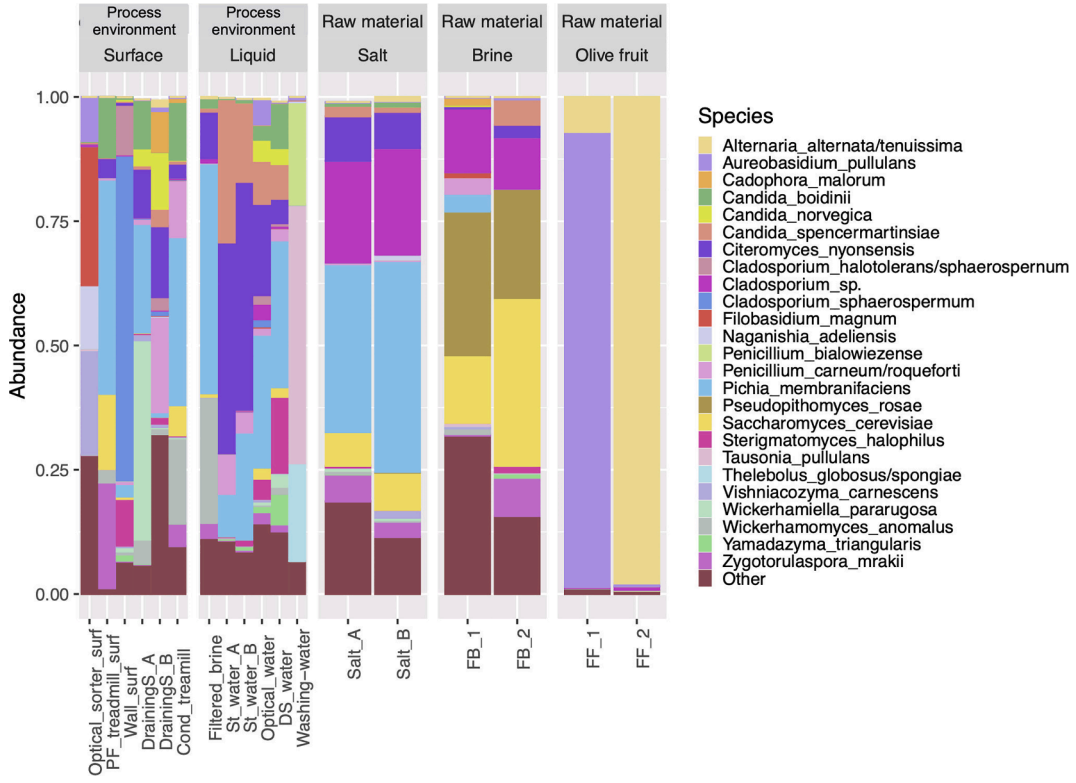

Fig.7. Potential contributors to fermentation fungal community based on SourceTracker analysis of ITS2 metagenetic sequencing data.

matrix type (brines versus olive fruits). Surprisingly, the second potential contributor that was identified corresponded to the draining system grids, especially for control fermentations. For these samples, in particular during the intermediary stages of fermentation (day 64 and day183 samples), stagnant water near the optical sorting treadmill was also identified as an important microbial source (2 to 70% depending on the samples). In general, fresh fruits did not seem to impact fermentation mycobiota and their contribution was restricted to three fermentation samples.

3.3.2. *Bacterial diversity analysis*

Bacterial composition was analyzed for process environment and raw material samples. Different profiles, based on the 35 most abundant species, are shown in Fig. 8. As illustrated, higher species diversity was clearly observed among process environment samples when compared to raw materials. More precisely, among process environment samples, up to 73 OTUs were identified in draining systems and 17 on the optical sorter surface, while <15 OTUs were identified in either raw materials (**Supplementary Table S1**). Regarding bacterial community profiles, although *C. diazotrophica* represented more than 80% of sequences in some samples (i.e. filtered brine, treadmill samples), high variability

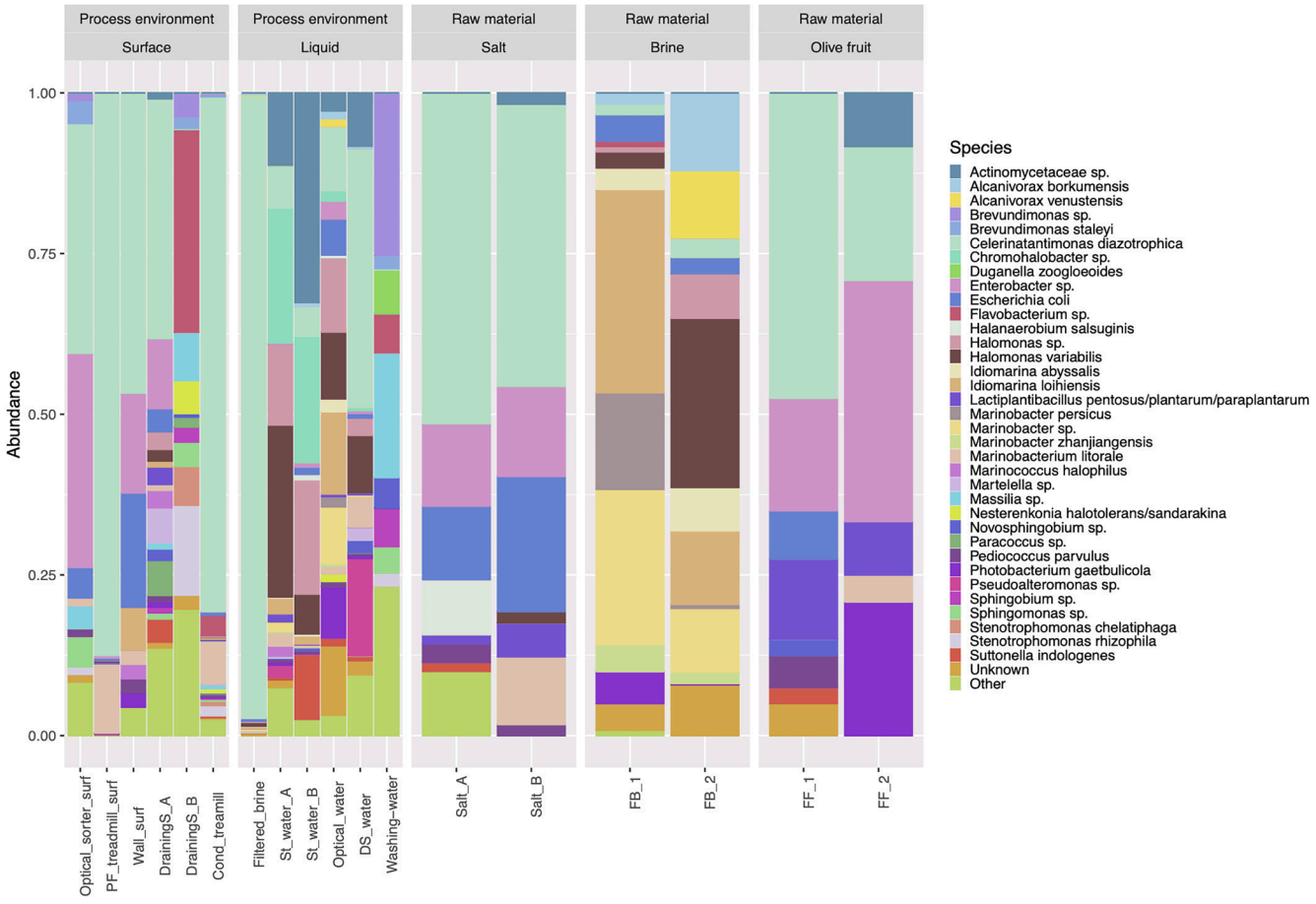

Fig. 8. Bacterial diversity (35 most retrieved species) encountered in raw material and process environment samples in the olive fermentation-plant as revealed by V3-V4 16S rRNA gene metagenetic analysis. For each sample, proportion of sequences affiliated at the species level are presented.

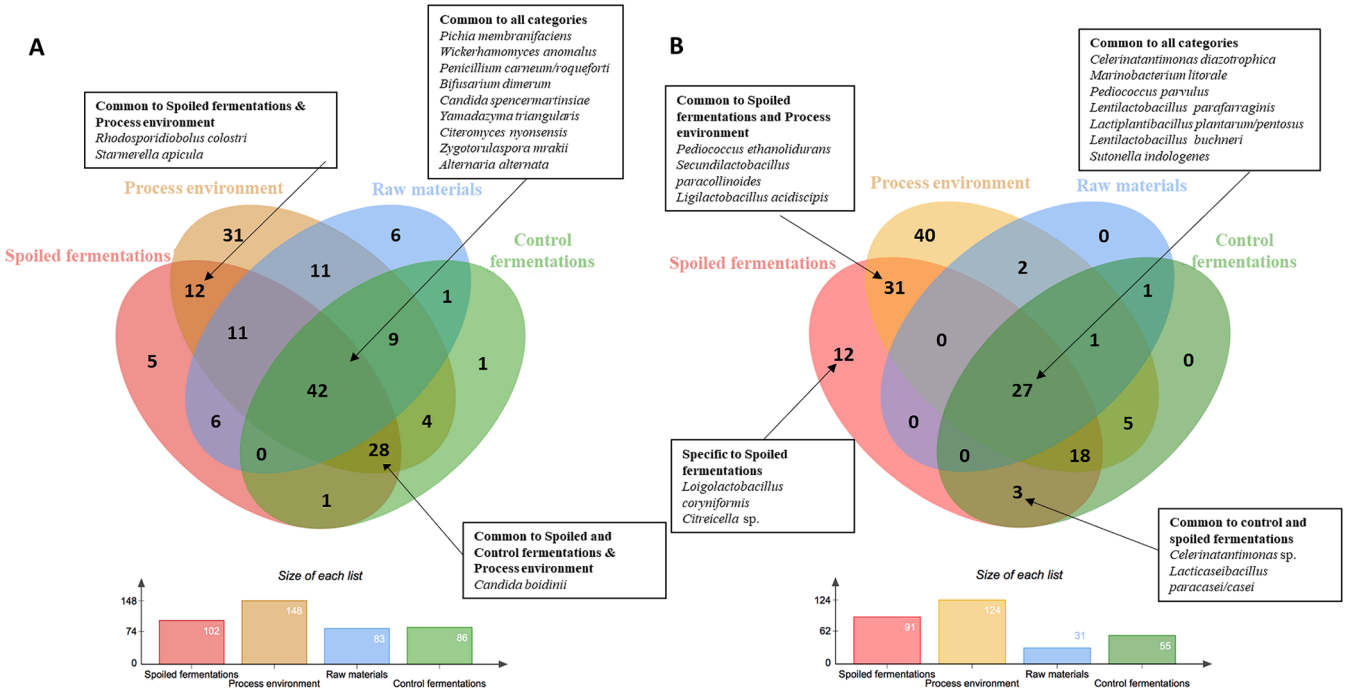

Fig. 9. Venn diagrams of **(A)** fungal and **(B)** bacterial distribution among fermentations and environment components. Numbers in each circle correspond to the specific or shared number of OTUs (affiliated at the genus or species level) obtained by metagenetic analysis. Dominant/major control fermentation or spoilage species position is highlighted in the boxes.

between surface and liquid samples was observed. Enterobacteria such as *E. coli* and *Enterobacter* sp., as well as other species belonging to the *Halomonas* and *Pseudomonas* genera, represented altogether between 26 and 40% of all sequences in most environmental samples. Bacterial diversity in raw materials was lower with <15 OTUs identified in salt samples and fresh olive fruits. Surprisingly, salt and fresh fruits had very similar profiles composed of *C. diazotrophica*, *Enterobacter* sp. and, to a lesser extent, *E. coli*; these three species being the most frequently identified in these samples. However, a main difference between both sample types was the presence of *Lactiplantibacillus pentosus/paraplantarum* in fresh olive fruits. In contrast, among raw material samples, higher diversity was observed in fresh brines as observed for *Idiomarina*, *Marinobacter* and *Alcanivorax* species.

SourceTracker was also implemented on 16S metagenetic data to determine potential bacterial sources in the process environment and among raw materials. However, most samples that could be considered as potential sources had insufficient read numbers to include them in the SourceTracker analysis. As a result, among the proposed microbial sources, none were identified to contribute to half of the studied fermentation samples (mainly spoiled samples) while stagnant water samples from the storage zones in the production facility were identified as the unique microbial contributor to all control fermentation samples (**Supplementary Fig. S4**).

3.4. Qualitative overview of environmental and fermentation microbiota

To study further the relationships between the in-house and spoiled and unspoiled olive microbiota, bacterial and fungal taxa (OTUs affiliated at the genus or species level) that were specific or shared among the different samples, i.e. process environment, raw materials, control fermentations and spoiled fermentations, were represented using Venn diagrams (Fig. 9).

Regarding fungal distribution (Fig. 9A), and as previously observed, highest diversity was found (represented by 148 OTUs) in process environment samples followed by spoiled fermentation samples (102 OTUs). In the case of raw materials and control samples, around 80 OTUs were observed when considering all samples in each group. Process environment was characterized by 31 specific OTUs, while for the other studied components, this number was lower. For instance, only 1 and 5 specific OTUs were observed in control and spoiled samples, respectively. When all components were considered, 42 OTUs were common. Interestingly, among these OTUs, the dominant yeasts (e.g. *W. anomalus, C. nyonsensis, Z. mrakii, C. boidinii*) were found throughout control fermentations as well as *P. membranifaciens, P. carneum* and *C. spencermartinsiae*, previously found in higher proportions in spoiled samples (see 3.1.2). In addition, 28 OTUs were found to be common to process environment samples as well as control and spoiled fermentations. Twelve other OTUs were only common to spoiled fermentations and process environment samples and included *Rhodosporidiobolus colostri* and *Starmerella apicula*. Both were found in significant abundances in the spoiled fermentation samples. Finally, 28 OTUs were shared by both control and spoiled fermentations and were mostly affiliated to the dominant species involved in control fermentations.

Regarding the distribution of bacterial species, as shown in Fig. 9**B, a** high number of OTUs (n = 40) were specific to process environment samples although they mainly belonged to subdominant species. On the other hand, no specific OTUs were identified for raw materials and control fermentation samples. Twenty-seven OTUs were shared by all four components and included bacteria found in control fermentations, namely *C. diazotrophica* and *M. litorale,* but also some LAB identified as dominant species in spoiled fermentations, especially *P. parvulus, L. parafarraginis, L. buchneri* and *S. indologenes*. Most interestingly, 31 OTUs were only shared between spoiled fermentation samples and those from the processing environment. Among these OTUs, *P. ethanolidurans, L. acidipiscis* and *L. paracollinoides* were identified. Finally, 12 OTUs (specific to spoiled fermentations) and three OTUs (shared by control and spoiled fermentations) were not found among the studied raw materials and environmental samples.

4. Discussion

Food spoilage is responsible for safety and quality defects that lead to important food losses for producers. In the case of traditional and artisanal fermented foods, process conditions are often less standardized than industrial ones, especially for small scale productions, and often involve indigenous microorganisms. These productions are thus more at risk to potential microbial contaminants and/or variations in microbial community structure (Capozzi et al., 2017). Microbial contaminations can be linked to raw materials or environmental origins although it should be emphasized that environmental sources can be both beneficial (potential reservoir of microorganisms for fermentations) or detrimental (food spoilers) (Hernández et al., 2018). In this study, both aspects were investigated for spoiled and unspoiled PDO Nyons table olives produced in the same facility.

Previous work on microbial dynamics during Nyons table olive fermentation revealed that the process was dominated by yeast populations and characterized by a rapid pH decrease in the first weeks of fermentation to around 4.4 (Penland et al., 2020). Table olives are most often linked to microbial spoilers that increase brine pH and this is typically caused by yeast or filamentous fungi growth (Lanza, 2013). On the contrary, in the present study, most spoiled fermented olives were characterized by a significant decrease in pH that was approximately 0.6 units lower than control fermentations as well as acidic sensory properties. These findings corroborated the microbial markers identified in bacterial communities. Indeed, high LAB counts were found in all spoiled fermentations while they were below the detection limit in control fermentations. Similarly, low levels of LAB were already observed in other tanks of Nyons table olives produced at two different harvesting times. Using 16S rRNA metagenetic analyses, diversified LAB populations were identified in spoiled olives and included *P. parvulus* and *P. ethanolidurans* in brines and *L. parafarraginis* (ex- *Lactobacillus parafarraginis*) and *L. buchneri* in olive fruits. These species undeniably dominated bacterial communities of spoiled samples and completely supplanted the species usually found, namely *C. diazotrophica* and *M. litorale.* It is not clear why these two species were inhibited in spoiled samples but a more acidic pH associated with higher organic acid concentrations as well as competition-exclusion could be responsible, among other factors, for this inhibition.

In most table olive preparations, LAB are usually considered as the main microorganisms responsible for the fermentation process and ensure rapid acidification in brines via lactic acid production (Hurtado et al., 2012). Some of the identified LAB species, such as *L. plantarum,* are commonly observed in table olives. *P. parvulus* was already identified in natural green olive fermentations after 6 months although as a subdominant species (Abriouel et al., 2012) as well as *P. ethanolidurans* (Lucena-Padrós et al., 2014). Nevertheless, in our study, their presence and known metabolism correlate with the observed sensory defects. Indeed, *P. parvulus* has been previously associated to wine-making and cider-making defects due to its ability to produce exopolysaccharides (Dols-Lafargue & Lonvaud-Funel, 2009; Ibarburu et al., 2010) and, in the current spoilage scenario, can be linked to the abnormal viscosity of spoiled brine samples (Wade et al., 2019). Regarding *L. parafarraginis* and *L. buchneri* species, their presence or spoilage activity has not been reported to date for table olives. However, these two heterofermentative LAB were associated with quality defects in cucumber fermentations (Franco et al., 2012). *L. buchneri* was shown to degrade lactic acid into acetic and propionic acids during cucumber fermentation (Johanningsmeier et al., 2012) and the same metabolism was reported for *L. parafarraginis* in silage (Xu et al., 2017). Considering the high levels of lactic acid previously quantified during Nyons table olive fermentations (Penland et al., 2020), this scenario is highly probable. Moreover, Johanningsmeier et al. (2012) further demonstrated that these

metabolic activities were highly dependent on pH but also salt concentration as lactic acid degradation increased as salt content decreased to 4 or 6% NaCl. For Nyons table olives fermented in brines, salt concentration is set to 10% as stipulated by PDO requirements. However, it is possible for this concentration to decrease over time as salt in brines slowly diffuses into olive fruits.

Regarding fungal communities in spoiled samples, counts were similar and the same species were identified although at different abundances. For example, *P. membranifaciens* dominated in most spoiled samples as well as several other filamentous fungi including *Penicillium carneum/roqueforti*, thus suggesting that these species were particularly adapted to the acidified environment. *P. membranifaciens* is known to grow at low pH, around 4 (Aguiar & Lucas, 2000), and is also resistant to high acetate concentrations (Oliveira et al., 2004).

Although some fungal species appear to be linked to spoilage in our study, complementary analyses would be necessary to confirm their spoilage behaviour (by artificially inoculating these species during fermentation) and to pinpoint the environmental determinants and outcomes. For the latter, salt quantification in brines could indicate if a strong deviation from usual salt concentration is the cause. Determination of volatile profiles of olive fruits could also be performed and compared to control fermentations in order to clearly identify spoilage biochemical markers. However, using the data obtained in the present study, source tracking analyses and the distribution of the different species in spoiled and control samples provided some leads.

Metagenetic analyses and source tracking were performed to identify potential microbial sources among raw materials, including fresh fruits, brine and dry salt, and process environment samples from different zones in the factory. Microbiota distribution comparison between these samples and spoiled, control fermentation revealed a "house microbiota" as the dominant yeast and bacterial species encountered during Nyons table olive fermentations were omnipresent in the process environment (on surfaces, draining systems), in brines and in dry salt. Surprisingly, fresh olive fruits only weakly contributed to both bacterial and fungal communities of Nyons table olives, although it was more pronounced for fungi than bacteria. Noteworthy, the highest diversity was observed in the process environment. Many OTUs and species, specific to these samples, showed that only a minority of microorganisms from the environment were able to settle in the fermentation. Interestingly, when comparing microbial distributions, most spoilage-associated LAB were present in raw materials (salt) and the process environment, although at low frequencies. *P. ethanolidurans* was only found in the process environment. Interestingly, the presence of these species in the environment in 2018 was not linked with any quality defect during fermentation. Although this cannot be generalized, the dominance of these species in spoiled fermentations further concurs with inappropriate fermentation conditions. It could be linked to the observed quality defects rather than punctual microbial contaminations. Noteworthy, the sources of some microorganisms remained unknown. This can be linked to either technical or sampling biases. Metagenetic analyses could be biased if too low sequence numbers are obtained to efficiently perform the analysis while a more thorough sampling campaign could be conducted to investigate other environmental sources.

Overall, the present study highlighted the positive and negative impact of the process environment and raw materials in shaping Nyons table olive microbial communities. On one hand, the revealed "house microbiota" was composed of the main microorganisms found in Nyons table olives. On the other hand, lactic acid bacteria, usually regarded as beneficial in table olive fermentations were associated with spoilage, and were found in low abundances in the environment. This further adds to the specificity of table olives microbiota existing from one preparation to another and further strengthens the importance of studying the in-house microbiota as potential microbial vectors linked to spoilage. In our case, it was clearly relevant to better comprehend fermentation outcomes, and provided more knowledge about these microbial vectors. Moreover, in the future, it could help adjusting the fermentation without compromising the traditional character of this process.

Data availability

Sequence data generated in this study are publicly available on the European Bioinformatics Institute database (www.ebi.ac.uk) under accession numbers **PRJEB40674** (fungal ITS2 sequencing run data) and **PRJEB40675** (bacterial 16S rRNA sequencing run data).

CRediT authorship contribution statement

MC, JM and SD obtained the funding and supervised the study. MC, JM and MP designed the experiments. MP performed the experiments and analyzed all the data except for SourceTracker analysis that was performed by JM. AP and ST provided technical assistance for microbiological analyses. MP drafted the manuscript and MC, JM and SD edited and proofread the manuscript.

All authors contributed to the article and approved the present version.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2021.110344.

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