**Yeast dynamics and volatilome analysis of naturally fermented Taggiasca black and green table olives**

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In the framework of this PhD project, the activities carried out included: the isolation and molecular identification of yeast colonies from 21-22 harvest to determine the ecology of fermented olives by using culture-based methodologies; 26S amplicon sequencing through the Illumina MiSeq platform to overcome culture-dependent limitations, and to deeply describe fermented olives mycobiota. Microbial ecology data was also complemented with the determination of volatile compounds via Gas Chromatography/Mass Spectrometry (GC/MS) analysis of both brines and olives samples.

Dinamica dei lieviti e analisi del volatiloma di olive Taggiasche da tavola verdi e nere fermentate naturalmente

Nell'ambito di questo progetto di dottorato, le attività svolte sono riportate di seguito. In un primo momento, è stato effettuato l’isolamento e l’identificazione molecolare degli isolati di lievito dalla raccolta 21-22. Successivamente, per superare i limiti delle metodiche cultura-dipendenti, il micobiota delle olive fermentate è stato analizzato tramite sequenziamento Illumina degli ampliconi del gene 26S rRNA. I dati molecolari sono stati inoltre complementati con la determinazione dei composti volatili effettuata con analisi Gas Cromatografia/Spettrometria di Massa (GC/MS) sia sui campioni di salamoia, che di olive.

**Key words**: Yeasts, spontaneous fermentation, Taggiasca olives, 26S Illumina sequencing, GC/MS.

# **1. Introduction**

According to the PhD thesis project previously described (Traina, 2022), this poster reports the main results of the following activities:

(A2) the culture-dependent analysis (microbial viable counts) over a 6-months fermentation period with the molecular data concerning the relative abundance of fungal species;

(A3) the culture-independent analysis of the total genomic DNA (gDNA) extracted from brines and olives;

Additional results that were not included in the previous project concern the determination of volatile compounds of both brines and olives samples for batch 1 and 2.

# **2. Materials and Methods**

*Culture-dependent analysis:* Taggiasca olives and brines samples deriving from two separate fermentation vessels were provided by a local company and were analyzed in duplicate from time 0 up until the end of the fermentation period (month 6). Sampling was performed at 0, 3, 7, 14, 28, 60, 90, 120, 150 and 180 days and a total of 373 yeast colonies were isolated and purified on Malt Extract agar (Generon, Modena, Italy) added with 0,5% (w/v) tetracycline (Fluka, Stainheim, Germany). For DNA extraction from isolates, 1 mL of a 24 h culture was transferred into 1,5 mL sterile microtubes (Resnova, Italy), filled with silica beads (VWR, Italy), and it was centrifuged at 13,000 x g for 5 min to pellet the cells. DNA extraction was carried out with the phenol:chloroform:isoamyl method according to Cocolin et al., 2004, and rep-PCR was carried out according to Versalovic et al., 1994, with the following modifications: initial denaturation at 95◦C for 1 min; 30 cycles at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. Dendrograms generation was performed with BioNumerics ver. 6.1 software (Applied Maths, Sint- Martens-Latem, Belgium) with the Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) for hierarchical cluster analysis. Isolates with similarity percentage > 85 % were considered to belong to the same cluster and one representative from each group was subjected to amplification of the D1/D2 loop of 26S rRNA gene with primer pairs NL1-NL4, and sequenced (GENEWIZ, Germany). The sequences obtained were compared with those on NCBI website, with Nucleotide BLAST search tool. *Culture-independent analysis:* Genomic DNA was extracted from brines and olives with the MasterPure complete DNA and RNA purification kit (Epicentre, Madison, WI) and it was used for the amplification of the D1 domain of the 26S rRNA gene with primer pair NL4R (5′-GGT CCG TGT TTC AAG ACG G-3′) and LS2-MF (5′-GAG TCG AGT TGT TTG GGA AT-3′), as described by Mota-Gutierrez et al., 2019. For data analysis, sequence adapters and primers were trimmed with *cutadapter* and DADA2 algorithm was used to filter raw reads. Classification of reads was performed using the DADA2 v.1.18 pipeline, and the Silva database was used for 26S taxonomy assignment.

# **3. Results and Discussion**

## **3.1 Culture-dependent results: abundance of yeast species**

The most dominant species overall were *Candida diddiensae* (36,02%), *Wickerhamomyces anomalus* (29,08%), *Aureobasidium pullulans* (13,87%) and *Pichia membranifaciens* (11,19%*).* In batch 1 the main species throughout the entire process were *W. anomalus* (46,19%), *C. diddiensae* (35,24%) and *A. pullulans* (6,67%), whereas in batch 2 the main representatives were *C. diddiensae* (33,74%), *A. pullulans* (26,38%) and *P. membranifaciens* (24,54%) (Figure 1).

 **A B**

*Figure 1:Relative abundances (%) of yeast species in olives (A) and brines (B) samples of batch 1 and olives (C) and brines (D) samples of batch 2. Data of brine at time = 3 days is not present because the sample was lost during transportation.*

Because of the persistence of yeasts throughout the entire fermentation process (3,34 and 5 Log CFU/g in olives and brines, respectively, by the end of fermentation), confirmed by yeasts counts, and the absence of lactic acid bacteria colonies on plate (<1 CFU/g in all sampling times which represents the detection limit), we can assess that this spontaneous fermentation was mainly driven by yeast populations. The main species identified were also in agreement with (Bevilacqua et al., 2012).

 **C D**

## **3.2 Culture-independent analysis**

26S rRNA gene sequencing showed a prevalence of *Aureobasidium* spp. (45,62%), *Citeromyces nyonsensis* (16,28%) and *W. anomalus* (13,05%),overall. As for batch 1, the main species found were *A. pullulans* (35,21%)and *W. anomalus* (42,99%)in olives and brines, respectively. In batch 2, the main representative species were *A. pullulans* (30,84%) and *C. nyonsensis* (67,33%) for olives and brines, respectively.

Comparing the two approaches, many differences were found in terms of detected populations in brines and olives.

Correlation with GC/MS data showed that the main ASV fraction, *A. pullulans* was positively correlated to heptanal, 1-hexanol-2-ethyl and ethanol (*P* < 0.05) and negatively correlated to 1-decanol-2-hexyl in olives (*P* < 0.05).

# **4. References**

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