A survey of bacterial and fungal communities of table olives.

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Abstract
Table olives are among the most ancient fermented foods. Their production extends beyond the Mediterranean basin and the transformation of different olive varieties according to various trade preparations results in high product diversity. Several varieties have Protected Designation of Origin, and, in Italy the black naturally fermented Oliva di Gaeta is prominent. In order to understand the diversity of bacterial and fungal communities and how it is affected by olive variety, olive ripeness and trade preparation, we carried out a survey using 364 samples from 40 producers and 6 countries. We measured pH, salt concentration, viable counts and carried out amplicon targeted metagenomics of bacterial and fungal communities. Olive variety, olive ripeness and trade preparation all significantly affected the diversity and composition of bacterial and fungal communities but dispersal plays a confounding role: even within the same variety we found significant differences between producing firms and a large variability even within the same firm. This may be due to limited control of contamination (with house microbiota playing a role in initial contamination events) and of technological factors and also to the widespread use of relatively small fermenters (240-280 L). This variability obscured the differences that existed between PDO (Oliva di Gaeta) and non PDO (Itrana nera) black naturally fermented olives of the Itrana variety. Nonetheless, we were able to identify subset of bacterial and fungal genera which were differentially abundant in selected trade preparations and confirmed that the diversity of lactic acid bacteria communities of naturally fermented olives.

Keywords
Table olives; amplicon targeted metagenomics; Oliva di Gaeta; olive fermentation; microbiota dynamics.

1. Introduction

Fermented foods have been part of the human diet for millennia and their diversity is huge (Gänzle, 2022; Hernández-Velázquez et al., 2024; Tamang et al., 2020). Fermented

vegetables are very diverse, and their production is often based on artisanal practices, without heat treatment of the raw materials and addition of starter culture, although back-slopping is frequently used (Tamang et al., 2020). Table olives are among the most ancient fermented vegetables from the Mediterranean basin (Langgut and Garfinkel, 2022) and this has resulted in a great variety of products (Anagnostopoulos and Tsaltas, 2022; Perpetuini et al., 2020). Fermentation of table olives has multiple functions: removing the bitter glucoside oleuropein, enhancing the shelf life through acid production in the brine, and improving sensory properties. Five main categories are recognized by the International Olive Oil Council (https://www.internationaloliveoil.org/wp-content/uploads/2019/11/COI-OT-NC1-2004-Eng.pdf). Alkali treated olives, also known as Spanish style olives, are treated with sodium hydroxide to remove oleuropein before being washed and fermented in brine. Green specialties, like Picholine style olives, on the other hand, are stored at low temperature in brine after the alkali treatment, thus limiting fermentation. Naturally fermented olives (Greek style) include both green and ripe olives directly fermented in brines.
Dried/shrivelled olives and olives darkened by oxidation, are preserved by methods other than fermentation. Several varieties have received Protected Denomination of Origin (PDO). In Italy four varieties (Oliva di Gaeta, Nocellara del Belice, Bella di Daunia, Oliva Ascolana del Piceno) are included in the PDO food list and the Oliva Taggiasca Ligure has recently received Protected Geographical Indication status (https://ec.europa.eu/agriculture/eambrosia/geographical-indications-register/). Many more are included in the Italian List of Traditional Agricultural Products (https://www.gazzettaufficiale.it/eli/gu/2024/03/13/61/sg/pdf). Spain, Greece, France, Portugal and Turkey also produce a wide variety of table olives, including PDO products.
Sensory quality, safety and stability of table olives are affected by several factors, including olive ripeness, technological factors, and by the growth of microorganisms, either present as natural contaminants on the fruits, salt, brines, and contact surfaces or added as starter cultures (Anagnostopoulos and Tsaltas, 2022; Campus et al., 2018). Current trends in table olive production aim at controlling the microbiota involved in fermentation, by sanitizing the raw materials, by adding starter and functional cultures, and/or by reducing salt content (Anagnostopoulos and Tsaltas, 2022; Campus et al., 2018). Addition of starters has been tested repeatedly and, typically, includes lactic acid bacteria (*Lactiplantibacillus pentosus* or *Lactipl. plantarum*, *Lacticaseibacillus paracasei* (Alfonzo et al., 2023; Comunian et al., 2017; Lanza et al., 2021, 2020; Paba et al., 2020; Pino et al., 2019, 2018; Randazzo et al., 2017; Rodríguez-Gómez et al., 2023; Ruiz-Barba and Jimenéz-Diaz, 2012; Servili et al., 2006; Tzamourani et al., 2024; Vougiouklaki et al., 2024; Zago et al., 2013)), yeasts (several species belonging to the genera *Candida, Wyckerhamomyces, Pichia, Cyteromyces* etc. have been tested: (Ciafardini and Zullo, 2019; Montaño et al., 2021; Ruiz-Barba et al., 2024; Tufariello et al., 2019)) or their combinations (Benítez-Cabello et al., 2020; Garrido-Fernández et al., 2021). Back-slopping practices (Martorana et al., 2015) have also been described.
The bacterial and fungal microbiota of table olives is complex, usually due to the lack of control of unit operations affecting dispersal and selection and includes microbial groups which are considered beneficial, like lactic acid bacteria, yeasts, and other halophilic microorganisms (Halophilic and Alkalophilic Lactic Acid Bacteria, HALAB, and halophilic Pseudomonadota), but also microorganisms associated with spoilage, like some halophiles (*Alkalibacterium*, *Marinilactobacillus*, *Celerinatantimonas*, some *Vibrio*, proprionibacteria, clostridia and yeasts (Arroyo-López et al., 2021, 2012; Ballester et al., 2021; de Castro et al., 2018; Penland et al., 2021) . The composition and dynamics of microbial communities in

table olives have been extensively reviewed (Anagnostopoulos and Tsaltas, 2022; Campus et al., 2018; Portilha-Cunha et al., 2020; Tsoungos et al., 2023; Vaccalluzzo et al., 2020). We have recently reviewed metataxonomic studies and found that a core microbiota of approximately 30 genera is characteristic of table olive fermentations, with wide variations among trade preparations and varieties (Ricciardi et al., 2025a). Geographic origin is also believed to be a contributing factor (Argyri et al., 2020; Kamilari et al., 2023).

With a few exceptions (Penland et al., 2022, 2021, 2020) studies on table olives microbiota are relatively small (<50 samples) and often focus on a limited number of varieties (see reviews by (Ricciardi et al., 2025a; Tsoungos et al., 2023) for details). This, and the variety of sampling plans and experimental approaches used, complicates the interpretation and the integration of these data to obtain a comprehensive view of the factors affecting table olive microbial community assembly and dynamics.

In this study, we carried out a large survey of table olives belonging to a wide array of varieties and trade preparations from 6 countries (Italy, Greece, Spain, Cyprus, Peru, Egypt) and including both sound and spoiled samples with the following objectives:

a. obtaining a comprehensive view of the bacterial and fungal microbial communities of table olives, understanding their variability due to ripeness and trade preparation, variety, between and within producing company.
b. using naturally fermented black Itrana olives as a case study to evaluate if detectable differences between PDO and non-PDO products exist

2. Materials and methods

2.1 Sampling.

Table olive samples (n=340) were obtained from 40 producers across 6 countries (Italy, Greece, Spain, Cyprus, Peru, Egypt). Producers were required to deliver olives ready for packaging in their fermentation brine, or immediately after packaging and to fill a questionnaire reporting essential metadata (location of the company, olive ripeness and variety, the techniques used for transformation, addition of starter cultures, if any; spoilage; duration of fermentation and of storage post fermentation, if applicable). Twenty sample, for which the fermentation brine was not available, were dispatched in the final packages, with the storage brine. Furthermore, 24 metagenomic DNA from table olives were obtained from Instituto de la Grasa, CSIC (Seville, Spain). Summary data are shown in Table 1, while detailed data are shown in Supplementary Table 1. All samples were dispatched in insulated containers with ice packs and reached our laboratory within 48 (samples from Italy) to 72 h. They were stored at 4°C for no more than 24 h before being used for microbiological analyses and extraction of DNA. Supernatants (12,000 x g) of the brine were stored at -20°C for 7-30 gg before the measurement of pH, titratable acidity and chlorides.

2.2 Chemical and physicochemical analyses.

pH and chlorides were measured in brines except for samples of dehydrated olives. The latter were homogenized (1:4) with deionized water in a Stomacher Lab Blender for 1 min using a filter bag, and pH and chlorides were measured in the filtered liquid phase.

pH was measured using a digital pH meter (CyberScan pH 110, Eutech Instrument Pte Ltd., Thermo Fisher Scientific, Monza, Italy) with a combination electrode (Double Pore Slim electrode, Hamilton Company, Reno, Nevada, USA). NaCl concentration was estimated from the measurement of chlorides on diluted (D = 10-50) brines using a selective electrode (Crison ISE Chlorid 51340400) with a reference electrode (Crison 5241 with KNO_3 1 mol/L as

142 a reference electrolyte), connected to an Orion 420 A plus ion-analyzer. 5 mol/L $NaNO_3$ was
143 used as ionic strength adjuster (1 ml / 50 mL diluted brine) and a calibration curve (0.001-0.1
144 mol/L NaCl)
145 Titratable acidity was only measured in brines using phenophthalein (Sigma) as an indicator
146 using NaOH 0.1 mol/L and a digital burette (Brand Titrette, Brand, Wertheim, Germany).
147 Two technical replicates were used for each measurement.
148
149

Table 1. The samples used in this project, by olive ripeness and trade preparation.

olive ripeness	olive trade preparation	n
Green olives	Alkali treated olives	68
Green olives	Natural olives	65
Green olives	Olives darkened by oxidation	5
Green olives	Specialties (Picholine, etc.)	30
Olives turning color	Natural olives	41
Olives turning color	Olives darkened by oxidation	9
Black olives	Dehydrated olives	4
Black olives	Natural olives	152

150
151 *2.3 Enumeration of microbial groups, isolation and identification.*
152 Counts were performed on a 1:1 mixture of olives (bones were aseptically removed) and
153 brine after homogenization (2 min) in a filtered bag (Nasco Whirlpak, Pleasant Prairie, WI)
154 using a Stomacher Lab Blender 400 (Cole-Parmer, now Antylia Scientific, Vernon Hills, IL,
155 USA) in peptone water (Bacteriological peptone 0.1% w/v, NaCl 0.85% w/v).
156 Serial ten-fold dilutions were performed in peptone water. Lactic acid bacteria were
157 enumerated in modified MRS agar (Lee and Lee, 2008; Ricciardi et al., 2014) with 0.02%
158 sodium azide (mMRS+NA, 25°C, 72 h), yeasts and moulds on Glucose Yeast Extract agar with
159 100 mg/L chloramphenicol (GYEAC, 25°C, 5 days), halophilic microorganisms on Plate Count
160 Agar Standard with 5% NaCl (w/v) (PCA + NaCl, 30°C, 48 h), contaminants on *Pseudomonas*
161 agar base (PAB, 25°C, 48 h), and *Enterobacteriaceae* in Violet Red Bile Glucose Agar (VRBGA,

30°C, 24 h). All microbiological media were inoculated using a spiral plater (WASP Spiral Plater, bioMérieux Italia SpA, Bagno a Ripoli, Italy) with the exception of VRBGA, for which conventional pour plating was used. Colony counts were performed using a digital colony counter (EasyCount 2, bioMérieux Italia).
Colonies were isolated and streaked for purification on the same medium and, after control of cell morphology by light microscopy, identification was carried out with MALDI-ToF Biotyper Sirius (Bruker Co, Mallerica, MA, USA) using the "Extended Direct Transfer" SOP provided by the producer.

2.4 Microbiological media, ingredients and reagents.
All microbiological media and ingredients were obtained from Oxoid (Basingstoke, United Kingdom), and reagents from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

2.5 Amplicon targeted metagenomics.
Olives (40 g) were mixed with an equal volume of brine (for dehydrated olives, sterile distilled water) to which Tween 80 (final concentration 0.1% vol/vol) was aseptically added. After shaking for 10 min on a rotary shaker (100 rpm) in ice, the liquid was centrifuged (8,000xg for 10 min) and the pellet was washed twice in sterile NaCl 0.85% and immediately frozen at -80°C. DNA extraction and purification were performed using DNeasy PowerFood Microbial Kit (QIAGEN Italia, Milan, Italy) followed by quantitation using NanoDrop One spectrophotometer (Thermo-Fisher Scientific, Milan, Italia). Library preparation and sequencing was performed by Novogene (Cambridge, UK). The V3-V4 region of the 16S rRNA gene was amplified using primers Bakt_341F_Nov (5'-CCTAYGGGRBGCASCAG-3') and Bakt_805R_Nov (5'-GGACTACNNGGGTATCTAAT-3'). The ITS2 region was amplified using primers ITS3F (5'-GCATCGATGAAGAACGCAGC-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') for Fungi. Paired end sequencing (2x250 bp) was performed on Illumina NovaSeq X.
Raw sequences were processed using a pipeline based on DADA2 (Callahan et al., 2016) using R (R Core Team, 2025). Model scripts are available at https://github.com/ep142/FoodMicrobionet/tree/master/dada2_pipeline. Taxonomic assignment was carried out using SILVA 138.2 (Quast et al., 2013) for bacteria and UNITE v10 general release 2024-04-04 for fungi ((Abarenkov et al., 2023). The results were assembled in a phyloseq object (McMurdie and Holmes, 2013) which was then used for further analysis. Extraction blanks and a mock community (ZymoBIOMICS Microbial Community DNA Standard, Zymo Research, Invine, CA, USA) were also included. Contaminating ASVs were removed using package "decontam" (Davis et al., 2018).

2.6 Statistical analysis
Non-parametric tests (Wilcoxon-Signed Rank Sum test) were used for comparing distributions of physico-chemical and chemical variables and counts (as log(cfu/g brine and olives)). Holm correction for multiple testing was used. Statistical analyses and graphs were produced with scripts combining the functions of the packages stats (R Core Team, 2025), PCAtools (Blighe and Lun, 2025), phyloseq (McMurdie and Holmes, 2013), ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2025) and rstatix (Kassambara, 2023). Alpha diversity analyses, ordinations and inferential analyses on microbial community data were performed using functions of the packages phyloseq (McMurdie and Holmes, 2013), DESeq2 (Love et al., 2014) and vegan (Oksanen et al., 2025).

3. **Results**.

3.1 Sampling.

A summary of the samples grouped according to ripeness and trade preparation is shown in Supplementary Table 1. Most of the samples (natural olives, 44%, Spanish-style, alkali treated Spanish-style olives, 19%) had undergone a fermentation step, while 27% had undergone an alkali treatment. The latter included both Spanish-style olives and specialties like Nocellara del Belice produced with the "Castelvetrano" method (Perpetuini et al., 2020). The remaining samples were produced with other methods, including drying. Spoiled samples were also made available by some producers (Supplementary Table 3).

3.2 pH, titratable acidity and salt content.

The pH of brines and olives in our study ranged from 3.7 to 8.1 (see Supplementary Table 4, Supplementary Table 5 for summary statistics). The high variability of pH of main varieties (those with at least 9 samples) is highlighted in Supplementary Figure 1.
The null hypothesis of lack of differences between the distribution of pH values (Wilcoxon Rank-Sum Test, WSRST, with correction for multiple testing) was discarded with $p < 0.001$ for the comparisons of black dehydrated olives and specialties (higher median pH) and the other groups. Even within a single variety, the pH was highly variable (see Supplementary Figure 1 and Supplementary Table 5). Results for titratable acidity (TA) of brines reflect those of pH with the exception of olives darkened by oxidation, to which gluconate was added. A box plot for the main varieties is shown in Supplementary Figure 2, while summary statistics are reported in Supplementary Table 6. After excluding olives darkened by oxidation and packaged olives, the titratable acidity ranged from 0.3 to 24 g lactic acid/L, with significantly higher median values (WSRT) for naturally fermented olives and lower values for alkali treated olives and specialties in particular. Again, high variability was observed even within the same variety. There was a weak (-0.58) but highly significant correlation between pH and the $\log_{10}$(TA).
Summary statistics for salt content for brines and olives are shown in Supplementary Tables 7 and 8. A box plot for the main varieties is shown in Supplementary Figure 3. NaCl content ranged from 2.2 g/L to near saturation (246 g/L) but median values ranged from 23 to 82 g/L for different combinations of ripeness and trade preparation. High variability was observed. For Oliva di Gaeta samples, which were the most numerous, NaCl content of brines varied between 30 and 116 g/L. Dehydrated olives and olives darkened by oxidation had significantly lower median values (WSRT).

3.3 Microbial counts.

Viable microbial counts were obtained using selective or elective media for undesirable microorganisms (*Enterobacteriaceae*, contaminants on *Pseudomonas* agar base) and potentially beneficial microorganisms. Based on MALDI-ToF or molecular identification of isolates (Supplementary Table 9) we found that both mMRS+NA and GYEAC were selective (all isolates were LAB or yeasts, respectively), while PAB (Supplementary Figure 5) supported the growth of yeasts (which formed pinpoint colonies) and PCA+NaCl supported both the growth of yeast and lactic acid bacteria together with staphylococci and other minor halophiles. The latter is therefore of limited use when counts of yeasts and LAB are high.

Counts of *Enterobacteriaceae* are shown in Supplementary Figure 4. For most samples counts were below the detection limit (<10 cfu/ml), while values > 100 cfu/g (brine + olives) were found in several samples of naturally fermented or alkali treated olives.
Counts of LAB are shown in figure 1 while summary statistics are shown in Supplementary Tables 10 and 11. Several samples (30%) had viable counts below the detection limit of our method (200 cfu/g), with 10^9 cfu/g detected as maximum counts. Spoiled samples of naturally fermented olives had almost always high counts of LAB, while this pattern was not observed in alkali treated olives. As for other variables, variability within variety was very high but some of them showed significantly higher (WSRT) counts compared to others (for example, Bella di Daunia vs Halkidiki for Green alkali treated olives; Kalamata and Konservolea vs Oliva di Gaeta for black naturally fermented olives). High counts of LAB were also found in alkali treated olives which are stored at low temperature after the alkali treatment, like Nocellara del Belice produced with the Castelvetrano method (Alfonzo et al., 2024).

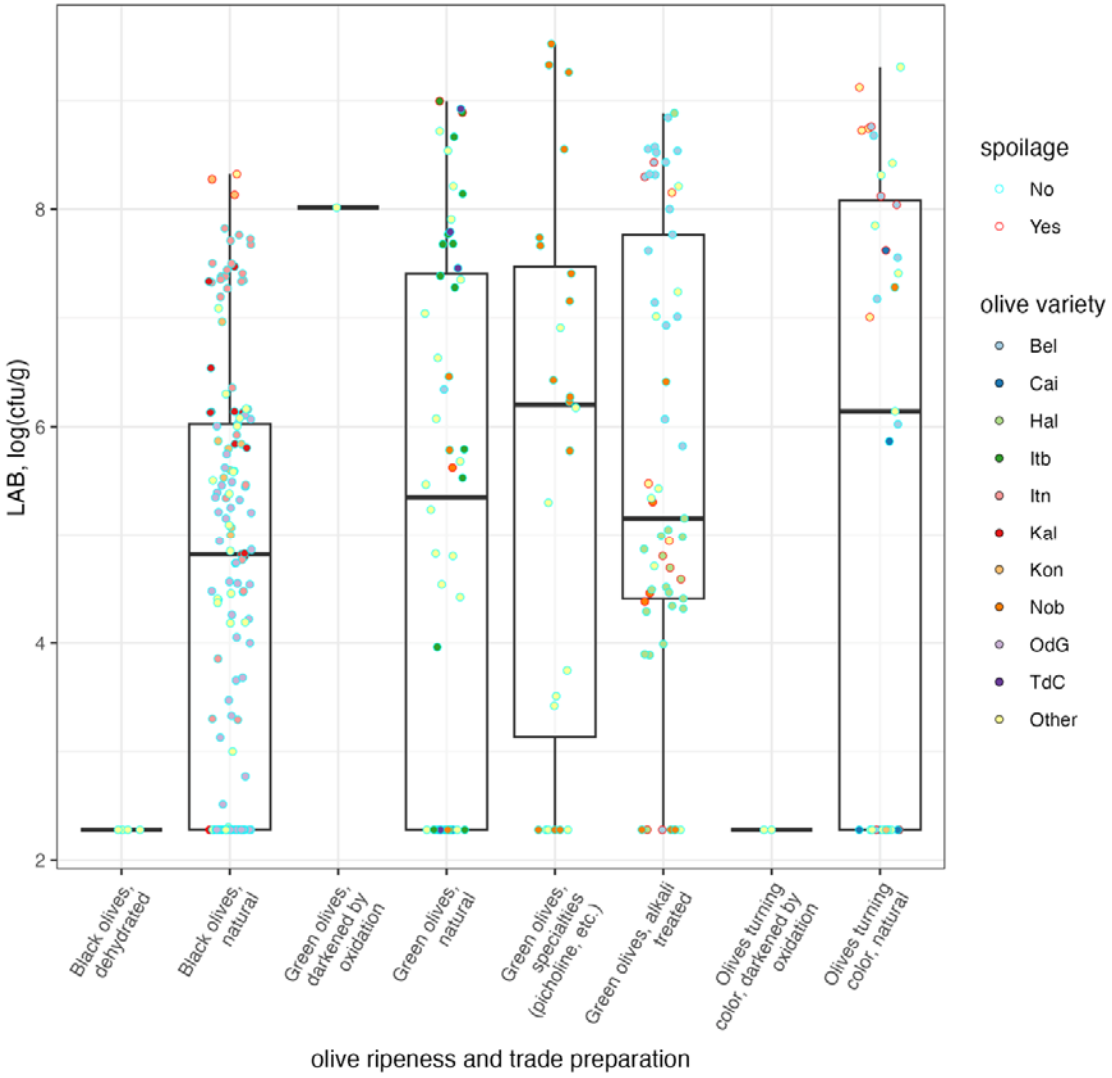


Figure 1. Distribution of viable counts of lactic acid bacteria in table olives used in this study.

Summary results on the counts of yeasts (moulds were only occasionally found) are shown in Supplementary Tables 12 and 13 and their distribution is shown in Figure 2. Only 6% of the samples had counts below the detection limit of our method and, for all olive groups, median values were higher than 10^5 cfu/g.

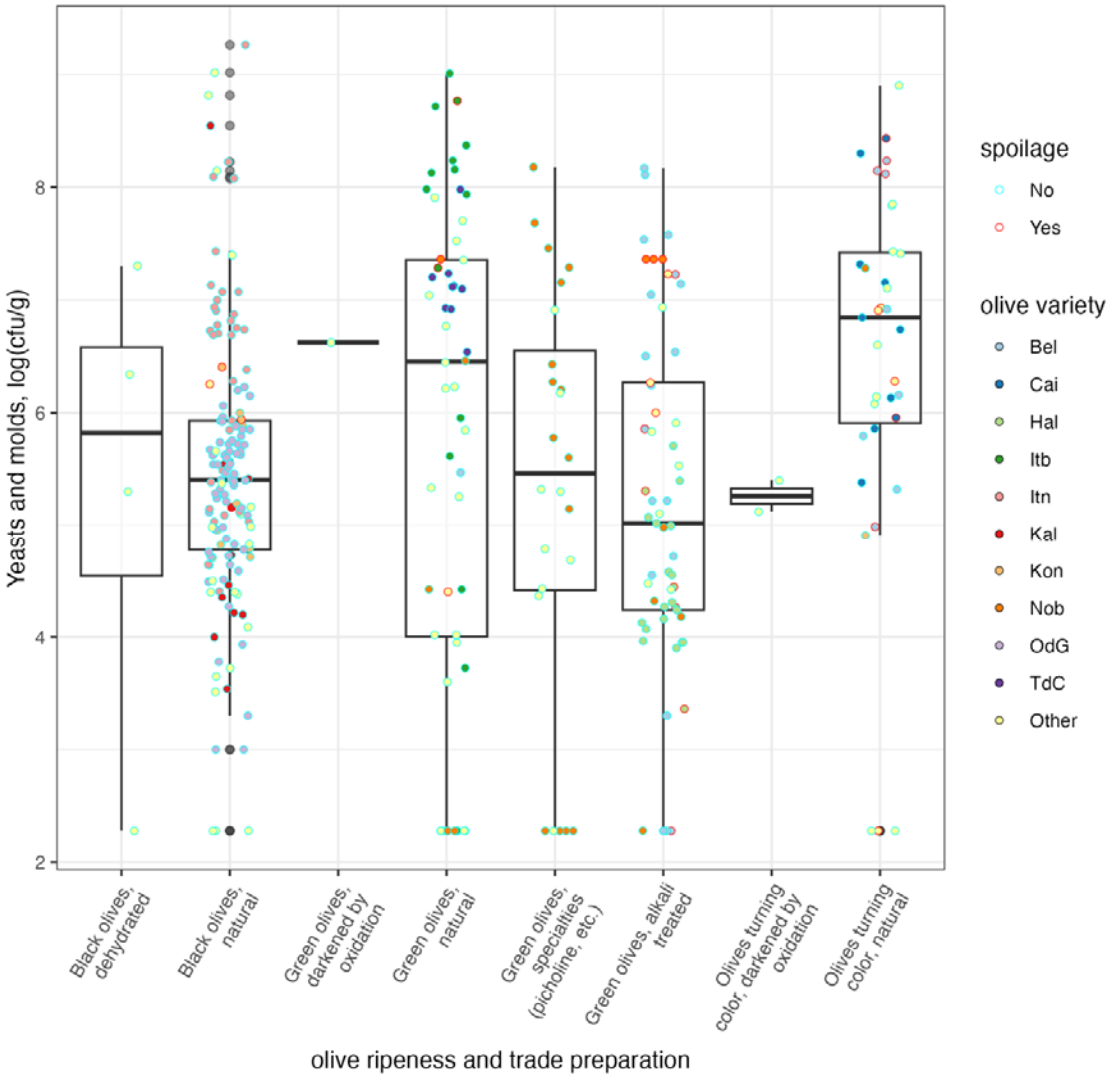
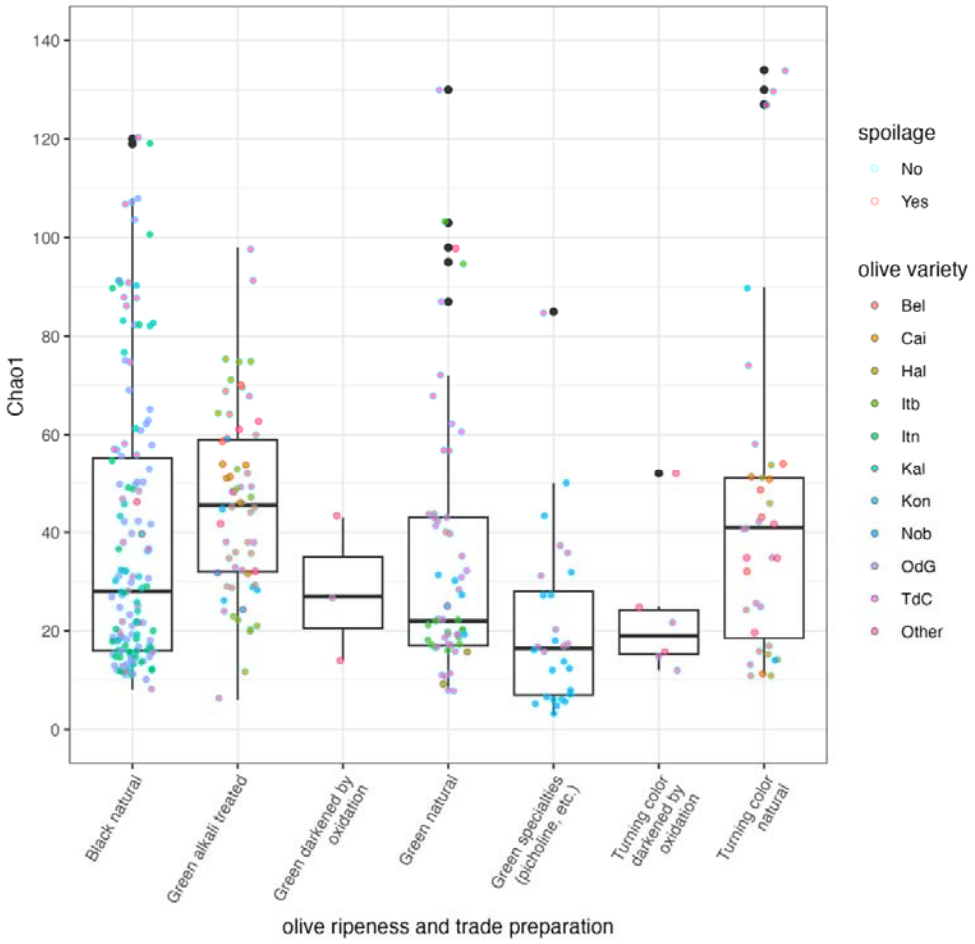


Figure 2. Distribution of viable counts of yeast and moulds in table olives used in this study.

Beyond the obvious negative correlation (Sperman rho -0.541) between pH and titratable acidity, absolute values of the Spearman rho correlation coefficient were often <0.3 (although many were significantly different from 0). As a result of the lack of correlation structure, the first two components of a Principal Component Analysis carried out on the correlation matrix explained only 43% of the variance, with no clear separation between different olive groups (see score plot in Supplementary Figure 6). This again, might be the result of the very high variability within groups.

289 **3.4 The composition of microbial communities of table olives**.
290
291 **3.4.1 Alpha-diversity of microbial communities.**
292 Extraction of DNA proved to be challenging for some low biomass samples, but we were
293 able to carry out sequencing of the V3-V4 region of the 16S rRNA gene (336 samples) and of
294 the ITS2 region (337 samples) of most of the samples. Mock communities (2) and blanks (3)
295 were included for quality control. The number of sequences after QC filtering, ASV inference
296 and bimera removal was $17.2x10^6$ for bacteria and $17.0x10^6$ for fungi. 90% of the samples
297 had 29.3 $x10^3$ sequences or more for bacteria, while the 90° percentile for fungi was 34.9
298 $x10^3$ sequences.
299 Our pipeline for 16S rRNA sequences theoretically allowed taxonomic assignment down to
300 the species level. However, this is not always possible for *Lactobacillaceae*, even when using
301 a custom database (Parente et al., 2023). Therefore, alpha and beta-diversity analysis for
302 bacterial communities was performed after agglomeration at the genus level. Chao1 values
303 calculated on the bacterial communities are shown in Figure 3, while summary statistics are
304 reported in Supplementary Tables 14 and 15.
305



306
307 **Figure 3.** Alpha-diversity (Chao1) of bacterial communities in table olives analysed in this
308 study.
309

310 Chao1 ranged from 3 to 134 (with median values for different groups ranging from 16.5 to
311 45). A large variability existed within each group and even within the same variety but some
312 trends were evident. If olive ripeness is ignored, there is a trend in the decrease of diversity
313 from Alkali treated olives, to natural olives, to olives darkened by oxidation, to specialties.
314 We decided to investigate if between-firm and within-firm variability was a contributing
315 factor. Distribution of Chao1 is shown for Itrana, Bella di Daunia and Halkidiki in
316 Supplementary Figures 7, 8 and 9. Within the same variety the diversity of bacterial
317 communities varied between different producers. We also evaluated the correlation
318 (Spearman ρ) between diversity and physical-chemical parameters and counts for selected
319 combinations of ripeness and trade preparation (Supplementary Table 16): with the
320 exception of a strong negative correlation between pH and diversity for Specialties (i.e.
321 diversity decreases as the pH increases possibly due to the selective effect of fermentation
322 occurring over time), all the other correlation coefficients were low (<|0.40|) although
323 highly significant and were not necessarily of the same sign for different trade preparations.
324 Again, differences exist among varieties, possibly because of systematic combinations of
325 specific factors affecting dispersal and selection: in fact, in Oliva di Gaeta, there was a strong
326 positive correlation (0.57) between pH and Chao1 compared to other black naturally
327 fermented olives (data not shown).
328 Given the larger sequence variability of ITS2 compared to 16S RNA V3-V4 sequences, we
329 decided to estimate diversity indices for fungi before agglomeration at the genus level. The
330 distribution of Chao1 values is shown in Figure 4, while summary statistics are in
331 Supplementary Tables 17 and 18.
332 Chao1 ranged from 1 to 209 (with median values for different groups ranging from 9 to 41,
333 lower than those for bacteria for most groups). As for other variables, a large variability
334 existed within each group and even within the same variety but some trends were evident: if
335 olive ripeness is ignored, the median value for Chao1 decreased from Specialties to alkali
336 treated olives, to naturally fermented olives. Significant differences among the distributions
337 were found using WSRT for the comparison between alkali treated olives (including
338 specialties) and natural olives. We investigated if between-firm and within-firm variability
339 was a contributing factor. As for bacterial communities, distribution of Chao1 values within
340 the same variety (Itrana olives, Bella di Daunia and Halkidiki olives: Supplementary Figures
341 10, 11 and 12) varied among different producers.
342 We also evaluated the correlation (Spearman ρ) between selected combinations of ripeness
343 and trade preparation (Supplementary Table 19): a rather strong negative correlation was
344 found with yeasts and molds counts and duration of fermentation. Again, differences exist
345 among varieties, possibly because of systematic combinations of specific factors affecting
346 dispersal and selection: in fact, in Oliva di Gaeta, there was a positive correlation (0.35)
347 between pH and Chao1; as for bacteria, higher pH is associated with higher diversity. On the
348 other hand, there was no significant correlation between Chao1 for fungi and bacteria.
349

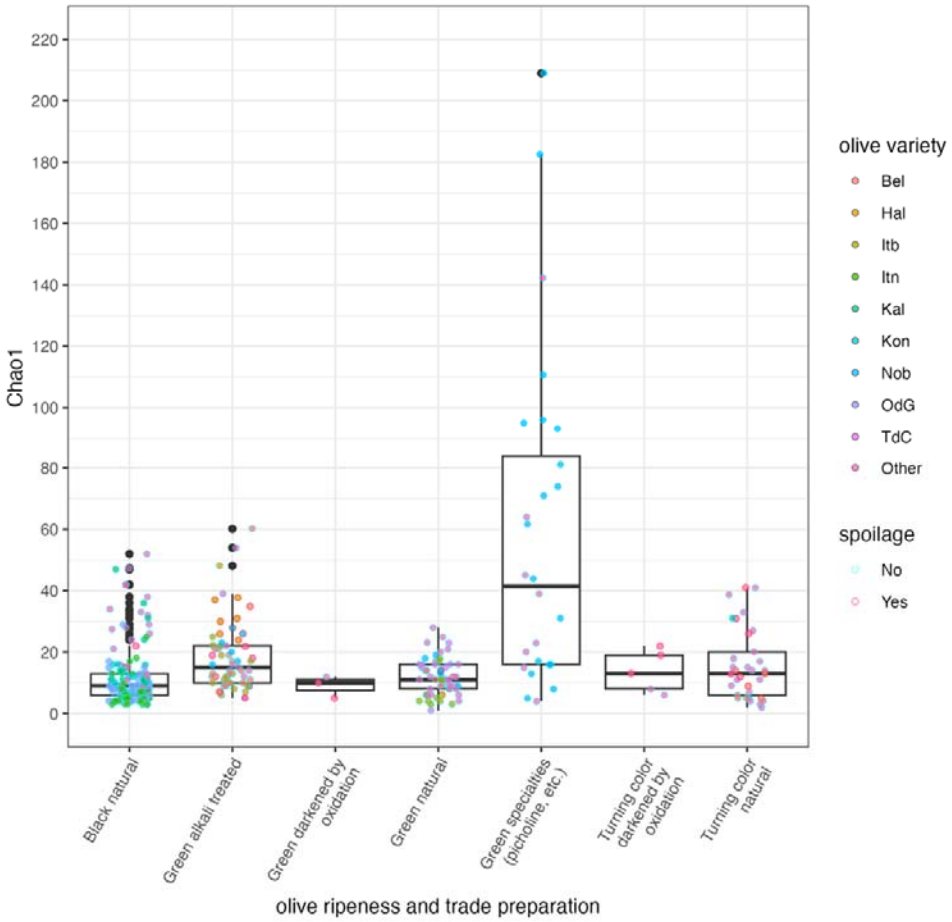


Figure 4. Alpha-diversity (Chao1) of fungal communities in table olives used in this study.

After prevalence and abundance filtering (> 1% maximum abundance and >1% prevalence), rare taxa were removed but >99.8% of the sequences were preserved for bacteria (Supplementary Figure 13). The top 24 bacterial taxa selected on the basis of abundance and prevalence and are shown in Supplementary Table 20. The large number of genera retained highlights the high microbial diversity found in table olives. The distribution of the abundance of LAB and HALAB is shown in Figure 5. Although abundances of the main genera are highly variable within each olive group, *Alkalibacterium* and *Marinilactibacillus* are abundant and prevalent in alkali treated olives, while a diverse set of *Lactobacillaceae*, among which *Lactiplantbacillus* is clearly both prevalent and abundant, dominating naturally fermented olives communities. Several heterofermentative LAB are often (*Lentilactobacillus*) or occasionally *(Limosilactobacillus*, *Levilactobacillus*, *Paucilactobacillus* and *Leuconostoc*) dominant or subdominant. It is interesting to note that, even in samples where *Lactiplantibacillus* was not dominant, *Lactiplantibacillus pentosus* was the most frequently isolated species anyway (Supplementary Table 9).
The distribution of halophilic genera is shown in Supplementary Figure 14. These included *Celerinatantimonas*, which was the most prevalent and abundant, but other Gram-negatives, such as *Halomonas, Vibrio, Salinicola* and *Idiomarina* were prevalent and occasionally abundant.
In a recent review we have found that DNA of genera including pathogenic bacteria can be found, albeit at very low abundance, in table olives (Ricciardi et al., 2025b). We repeated the

analysis for this survey and found that only in very few samples sequences assigned to genera *Salmonella, Yersinia* and *Listeria* were detectable, usually at very low abundance, with one notable exception (Supplementary Figure 15).

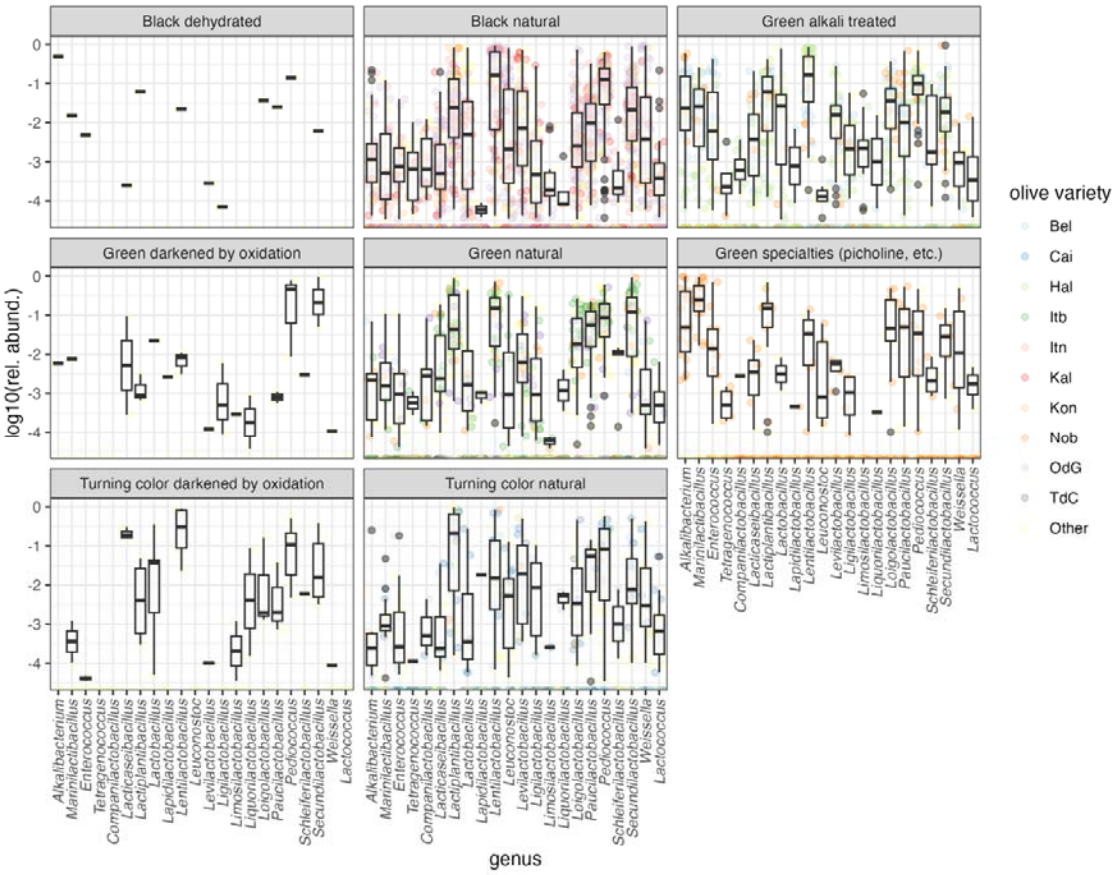


Figure 5. Distribution of the relative abundance of LAB and HALAB in table olives. Genera are ordered by alphabetical order within family.

Bar plots of the relative abundances of the 24 most abundant and prevalent genera are shown in Supplementary Figures 16 (pooled data for all varieties, by ripeness and trade preparation group) and 17 to 21 (main varieties within ripeness and trade preparation groups). Some general patterns are evident: more frequent and abundant occurrence of HALAB in alkali treated olives, while LAB, mainly *Lentilactobacillus, Lactiplantibacillus* and *Pediococcus* were dominating members in naturally fermented olives. However, variety-specific patterns appear. Two examples, for Bella di Cerignola (used for different variations of Bella di Daunia PDO) and Itrana (used for the production of two non-PDO types and of Oliva di Gaeta PDO) are shown in Supplementary Figures 22 and 23. In both cases, while there is some similarity in the composition of the bacterial community within a given variety, differences between different firms are visually evident.

Abundance and prevalence filtering was performed on fungal communities using approaches similar to those described above. Our pipeline allowed the assignment at the species level for the vast majority of ASVs, but given the debate on the reliability of species assignment using regions of the ribosomal RNA operon in fungi (Kauserud, 2023; Rué et al.,

2023; Tedersoo et al., 2022) we decided to perform the analysis both at the species and genus levels. Abundance and prevalence filtering at the species level retained 195 taxa out of 795, with 99.6% of sequences, mostly belonging to phylum Ascomycota (Supplementary Figure 24). Twenty-nine taxa had a prevalence > 0.1 and a maximum abundance > 0.01 and may be considered as the core mycobiota of table olives (Supplementary Table 10). Six species (*Pichia membranifaciens, P. manshurica, Candida boidinii, Dekkera custersiana, Wickerhamomyces anomalus, Saccharomyces bayanus*) have a prevalence >0.5 and a maximum relative abundance >0.90, but with one exception, their median abundance is <0.01: in fact, they may dominate some samples but may have a low abundance in others (see Supplementary Figure 25 for a boxplot of the distribution of core species).
The most abundant species were all cultivable, and were in fact isolated from counting plates (Supplementary table 9).
To simplify the analysis, we performed aggregation at the genus level. Core fungal genera are shown in Supplementary Figure 26 and, remarkably, they largely overlap with those found in a recent review (Ricciardi et al., 2025b). To avoid losing genera which are important only in one group of olives we used the same approach used for bacterial communities (the union of the sets of taxa which passed the filter in each group was retained): this resulted in 107 genera. Figure 6 shows a box plot of the most important families (*Pichiaceae, Saccharomycetales_fam_Incertae_sedis* and *Phaffomycetaceae*), while other genera are shown in Supplementary Figures 27 and 28.
As for bacterial genera there is a very wide variability within each ripeness and trade preparation group and sometimes even within the same variety. However, some patterns appear to emerge: *Pichia* is by far the most abundant and prevalent genus in all types of olives which have undergone a fermentation (including green alkali treated olives) while the distribution of other important genera is more variable and often clearly multimodal, with very high abundance in some samples and very low in others. Specialties clearly have the largest variety of fungal genera, possibly due to the lower duration of fermentation and the less selective conditions.
A summary bar plot with the relative abundance of the main fungal genera in ripeness/trade preparation groups is shown in Supplementary Figure 29. Overall, *Pichia* dominated most of the groups, and the sum of relative abundance of *Pichia, Candida, Wickerahmomyces, Saccharomyces, Zygotorulaspora* and *Dekkera* usually exceeded 75%. On the other hand, there are notable differences among varieties within each group (Supplementary Figures 30-34). The high fungal diversity in specialties, even within the same firm (most samples of Nocellara del Belìce were provided by one firm over two years) is evident from Supplementary Figure 33. While the composition of fungal communities of samples of the same combination of olive variety and trade preparation is remarkably similar, samples from some firms have a substantially different composition (Supplementary Figures 35-36).

3.4.2 Beta-diversity of bacterial and fungal communities.

In order to compare the microbiota of individual samples we used different ordination approaches on the Bray-Curtis distance matrix. A non-monotonic Multidimensional Scaling for bacterial communities in three dimensions resulted in a low stress (0.13) and the configuration for the first two is shown in Figure 7.

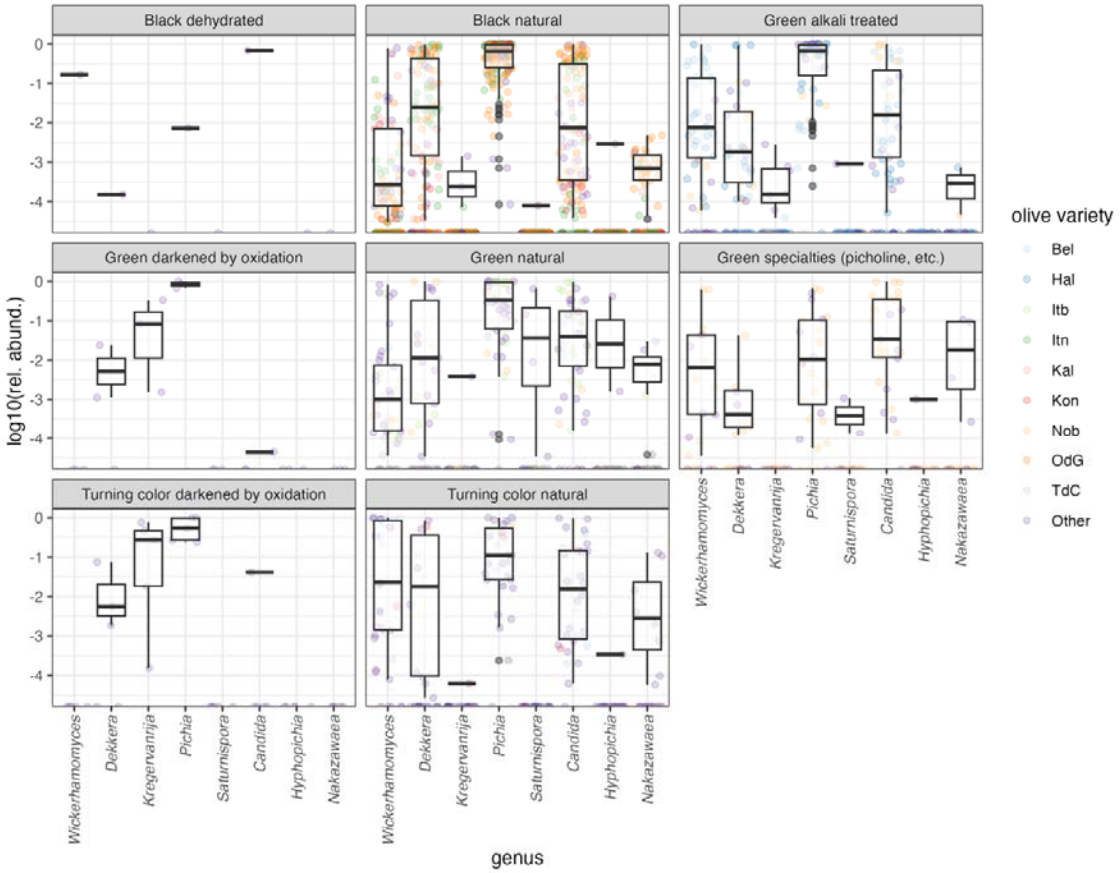


Figure 6. Distribution of fungal genera belonging to families *Pichiaceae, Saccharomycetales fam Incertae sedis* and *Phaffomycetaceae* in table olives used in this study. The main varieties are shown (see Supplementary table 2 for abbreviations).

The variability within each group is evident, although groups tend to occupy a specific area of the graph. No clear pattern for the clustering of spoiled samples (which were often but not always far from non-spoiled samples) is evident, but this may be due to the variety of spoilage types in our dataset. The between-firms and within-firms variability was substantial: selected examples are shown in Supplementary Figures 37-39.
Simple ordination plots are insufficient to clearly visualize differences among varieties and this may due to the inability to separate variance associated with predictors (chemical and microbiological analyses, ripeness/trade preparation groups) from that due to random variation. When constrained ordination approaches, like Canonical Correspondence Analysis ordination using quantitative variables (microbial counts, chemical analyses) were used, only a very low amount of the variance was explained by the first components.
However, inferential methods (PERMANOVA with adonis2) showed that a general pattern existed and that the combination of ripeness and trade preparation significantly (0.001) affected the composition of the bacterial communities. The effect of olive varieties within a gives ripeness and trade preparation was also significant for all main combinations except for turning color naturally fermented olives (Supplementary Table 22). For selected varieties more than one producer with at least 5 samples were available: we therefore tested the hypothesis that, within a given variety, producing firm was not a contributing factor to the

composition of bacterial communities: the null hypothesis was discarded for all the varieties tested (Itrana nera, Kalamata, Bella di Daunia and Halkidiki) (Supplementary Table 23).

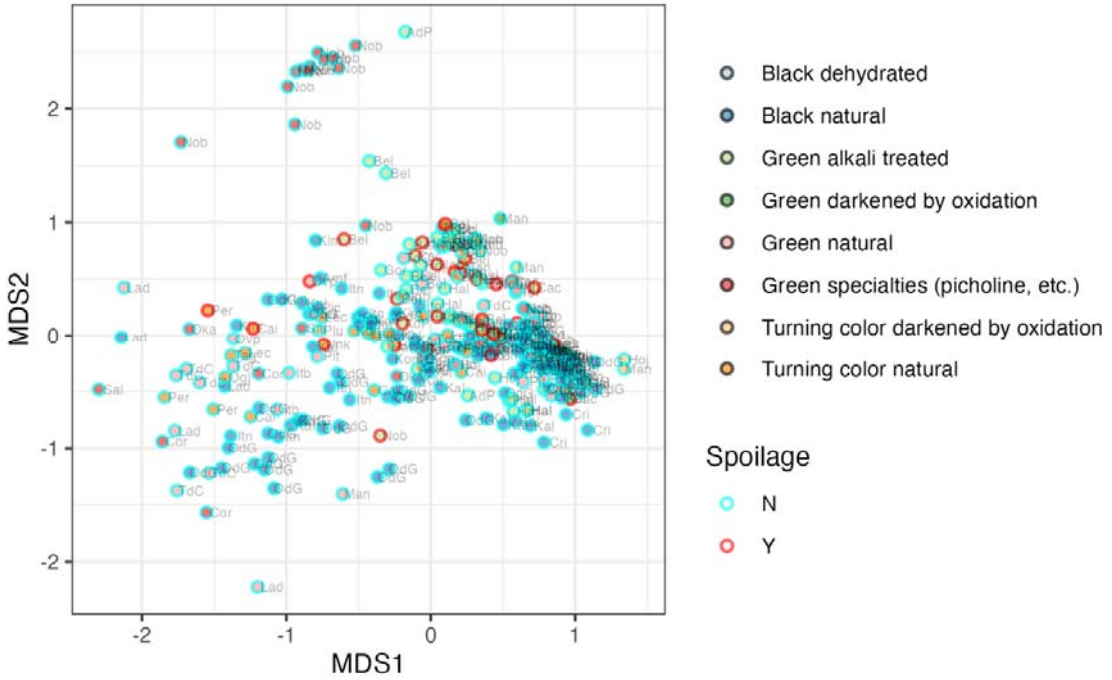


Figure 7. Configuration plot for the first two dimensions of a non-monotonic Multidimensional Scaling of the Bray-Curtis distance matrix of the composition of bacterial communities of table olives. See Supplementary table 2 for abbreviations of olive varieties.

We finally explored differentially abundant taxa in olive ripeness / trade preparation groups using DeSeq2 with alpha = 0.01, and then created contrasts for comparing groups of olives. The results are shown in Supplementary Figures 40 to 47 and in Supplementary Table 24. The differentially abundant genera were not necessarily those with high relative abundance (see Figure 5 and Supplementary Figures 16-23) but the patterns found in alpha-diversity and inferential analysis clearly emerge. HALAB and, in general alkalophilic microorganisms, are significantly more abundant in alkali treated olives; several Pseudomonadota are typically more abundant in black and turning color naturally fermented olives. Heterofermentative genera, like *Levilactobacillus*, *Leuconostoc* and *Weissella* are more abundant in black natural olives compared to all types of green olives. The pattern for *Lactobacillaceae* is more variable. *Lactiplantibacillus* is significantly more abundant in black natural olives than in alkali treated green olives (including specialties), but *Lacticaseibacillus* is more abundant in Green natural compared to Black natural olives. Overall, a variety of homofermentative and heterofermentative *Lactobacillaceae* are significantly more abundant in naturally fermented olives compared to alkali treated olives.
Finally, we used PERMANOVA to test the null hypothesis of lack of differences in the structure of bacterial communities for Itrana nera and Oliva di Gaeta (its PDO counterpart). The null hypothesis was rejected with $p = 0.025$. However, this result might be due exclusively to samples provided from one firm (F23 provided 90% of the samples for these varieties) and might be difficult to confirm if more samples were added from the same or other firms.

Non-constrained ordination for fungal communities was carried out using the same approach used for bacterial communities and the configuration for the first two dimensions is shown in Figure 8. The only group which is clearly separated is that of specialties. To visualize more clearly the effect of spoilage and the within-group variability the same configuration is shown separately for Black natural, Green alkali treated and Green natural olives in Supplementary Figures 48-50. There is sometimes a large variability for the same variety produced by different firms (this is evident for both Oliva di Gaeta PDO (Supplementary Figure 48), and other varieties, like Halkidiki and Bella di Daunia (Supplementary Figure 49). Samples produced by the same firm tend to be close among them, but this not always true. For some varieties spoiled samples are clearly different from unspoiled ones in terms of composition of fungal communities. Unfortunately, as for bacterial communities, we do not have enough spoiled samples nor a balanced distribution for defects to draw any specific conclusion.

Constrained ordination explained a negligible amount of variance (results not shown).

We also used PERMANOVA with adonis2 to test the null hypothesis that combination of ripeness and trade preparation did not affect the composition of fungal communities (which was rejected with p=0.001) and that olive varieties (within the main combinations of ripeness and trade preparation) did not affect composition of fungal communities, which was rejected for all main groups (Supplementary Table 25). For the three varieties (Oliva di Gaeta, Kalamata and Bella di Daunia) for which we had more firms, we also tested the hypothesis that there were no differences among the firms: the null hypothesis was rejected for Kalamata and Itrana Nera, Halkidiki, but not for Bella di Daunia (Supplementary Table 26). Both findings support the hypotheses that while selective factors related to variety and technology are important, house microbiota and specific practices within firms also play an important role in the assembly of fungal communities.

We also performed PERMANOVA to test the null hypothesis of lack of differences in the structure of fungal communities for Itrana nera and Oliva di Gaeta (its PDO counterpart). The null hypothesis was rejected with p = 0.002. Again, this result might be due exclusively to samples provide from firm F23.

We then carried out differential abundance analysis using DeSeq2 with alpha = 0.01, and then created contrasts for comparing groups of olives. The results are shown in Supplementary Figures 50 to 57 and in Supplementary Table 27. The differentially abundant genera were not necessarily those with high relative abundance (see Figure 7 and Supplementary Figures 33-34) but the patterns found in alpha-diversity and PERMANOVA analysis emerge.

For example, many filamentous fungal genera appear among those which are differentially abundant in one or more contrasts but their relative abundance is often very low. In other cases (the yeast *Trichosporon* is an example) the significance in the differential abundance test simply depends on the absence of a genus in some groups. Given the low abundance it is likely that these species originate from the carposphere environment and do not grow well during fermentation. If we limit the analysis to the most abundant and prevalent core genera, we confirm the patterns found with alpha and beta-diversity analyses.

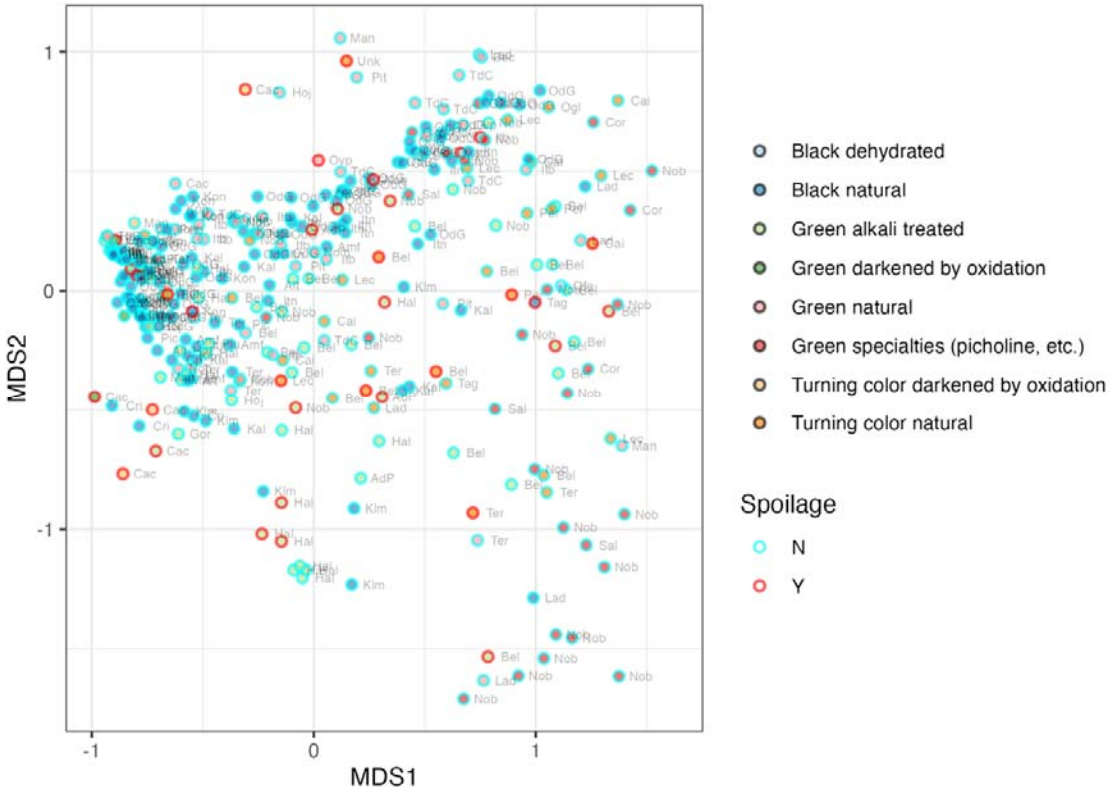


Figure 8. Configuration plot for the first two dimensions of a non-monotonic Multidimensional Scaling of the Bray-Curtis distance matrix of the composition of fungal communities of table olives. See Supplementary table 2 for abbreviations of olive varieties.

Schwanniomyces and *Geotrichum* were more abundant in Green alkali treated olives that in any type of naturally fermented olives. *Wickerhamomyces* was significantly less abundant in black natural compared to Green and Turning color natural and Green alkali treated olives. *Dekkera* was found to be more abundant in black and turning color natural compared to specialties. *Pichia* was more abundant in black and turning color natural compared to specialties, but also in green alkali treated compared to green natural: this may in fact reflect differential abundance of species within this genus (see Supplementaty Figure 25). *Citeromyces* was more abundant in all types of naturally fermented olives compared to green alkali treated olives. *Kluyveromyces* is more abundant in alkali treated compared to naturally fermented olives. *Saccharomyces* abundance was significantly higher in green natural olives but this result may be affected by the very high relative abundance of this genus in all Tonda di Cagliari samples. The abundance of the genus *Candida* was lower on average in black natural compared to both green alkali treated and green specialties: however, this may not be true for all varieties and all species, since *C. boidinii* was very abundant in some samples of black natural olives (see Supplementaty Figure 25).

4. **Discussion**.

In this work, we have presented what is, to date, the largest survey on the composition of bacterial and fungal communities of table olives.

563 Our main emphasis was on olives produced in Italy, including PDO varieties such as Ascolana
564 del Piceno, Bella di Daunia, Nocellara del Belìce, and, especially, Oliva di Gaeta
565 (Supplementary Table 1). The latter is produced using black Itrana olives, with a long natural
566 fermentation process and, together with its non-PDO counterpart (Itrana nera), is one of the
567 most important table olive varieties in Italy, but studies on its microbiology are
568 comparatively rare and have used traditional cultivation-based approaches. Although some
569 authors have reported high numbers of LAB and yeasts in pulp and or/brine (Sacchi et al.,
570 2022; Tofalo et al., 2012) others have reported low LAB abundance in uninoculated
571 fermentations (Servili et al., 2006) . Addition of starters (*Lpl. plantarum/pentosus* with or
572 without yeasts) has been reported to improve the control of fermentation (Lanza et al.,
573 2021, 2020; Servili et al., 2006) . However, to our knowledge, no metataxonomic or
574 metagenomic studies on the microbial communities of naturally fermented Itrana olives are
575 available.
576 Given the heterogeneity of olive variety, production processes and the limited control
577 artisanal or semi-industrial producers have on key factors affecting contamination and
578 microbial community dynamics (Anagnostopoulos and Tsaltas, 2022; Perpetuini et al.,
579 2020) , variability among products belonging to the same trade preparations or even within
580 the same variety is expected, but the extent of this variability has never been explored in
581 great detail and attempts at relating factors like origin or trade preparation with microbiota
582 are rare (López-García et al., 2025) .
583 Indeed, almost all the olives fermented under brine in our dataset were obtained from
584 plastic fermenters (240-280 L) left at uncontrolled temperature. This inevitably leads to a
585 lack of control of the fermentation and high variability in the microbial communities.
586 Addition of starter cultures has often been shown to result in more uniform quality and
587 more rapid acidification (Benítez-Cabello et al., 2020; Campus et al., 2017; Giavalisco et al.,
588 2024; Leal-Sánchez et al., 2003; Vaccalluzzo et al., 2022) and should be encouraged,
589 provided suitably competitive cultures are available.
590 pH and salt content, together with nutrient availability and polyphenol contents are among
591 the most important ecological factors affecting the survival and growth of microorganisms in
592 table olives (Campus et al., 2018; Tsoungos et al., 2023) with NaCl acting as the main factor
593 controlling the early growth of undesirable microorganisms (Perpetuini et al., 2020).
594 It is interesting to note that many of our samples have combinations of pH, titratable acidity
595 and NaCl which are less inhibitory for microorganisms than those indicated in the trade
596 standards for packaged olives (IOC, 2024): this is not surprising, since one of the criteria of
597 inclusion in our study was the delivery of samples in their fermentation brine at or past the
598 time at which the fermentation was deemed to be completed. Replacement of the
599 fermentation brine with a preservation brine containing organic acids is common at
600 packaging (IOC trade standards, IOC, 2024). However, we did not find any indication that
601 spoilage was related to pH, TA or salt content for individual varieties.
602 We used a combination of selective and differential culture media for counting LAB, yeast
603 and molds, halophilic and potentially deleterious microorganisms (*Enterobacteriaceae* and
604 other contaminants). Only mMRS+NA and GYEAC showed sufficient selectivity for the
605 enumeration of LAB and yeasts, respectively. MRS with sodium azide is frequently used for
606 the enumeration of LAB in table olives (Benítez-Cabello et al., 2020; Castro et al., 2018;
607 Sánchez et al., 2018) although other inhibitors, like actidione (Argyri et al., 2020; Kazou et
608 al., 2020; Maoloni et al., 2022; Tzamourani et al., 2021) and natamycin (Anagnostopoulos et
609 al., 2020) have been used to make MRS selective. PCA+5% NaCl may be of limited use for the

610 counts of halophilic microorganisms when the counts of LAB and yeasts are high because
611 both groups may grow on this medium. With the exception of PAB these media are
612 commonly used for enumeration of microorganisms in table olives (see (Benítez-Cabello et
613 al., 2020; Castro et al., 2022; Penland et al., 2020) for examples) but we are not aware of
614 scientific papers questioning their selectivity. LAB counts were highly variable in our samples
615 and, often, they were below the detection limits of our method. LAB are among the most
616 important members of table olives microbiota (Portilha-Cunha et al., 2020). High counts in
617 brine are commonly found at the beginning of fermentation, although they can decline over
618 time (Benítez-Cabello et al., 2020; Sánchez et al., 2018) and especially in high salt brines,
619 LAB have not been found with either culture based or culture independent approaches
620 (Portilha-Cunha et al., 2020; Traina et al., 2024).
621 High yeast counts were more consistently found. This is common in olives and brines and
622 the role of different species of yeasts can be either beneficial or detrimental, although they
623 are frequently included in starter cultures (Anagnostopoulos and Tsaltas, 2022; Arroyo-López
624 et al., 2012; Giavalisco et al., 2024) where is has been shown that they can stimulate the
625 growth of LAB (Benítez-Cabello et al., 2020).
626 *Enterobacteriaceae* are commonly found at the beginning of fermentation in table olives but
627 disappear as pH becomes lower. However, genus *Enterobacter* has been found to be both
628 prevalent and abundant in table olives (Ricciardi et al., 2025b) and high counts of
629 *Enterobacteriaceae* have been occasionally found in naturally fermented olives
630 (Anagnostopoulos et al., 2020; Maoloni et al., 2022; Penland et al., 2020). In our study
631 metataxonomic analysis revealed that DNA of several genera of *Enterobacteriaceae* was
632 present in high proportions in several varieties even at the end of fermentation, but it is
633 unclear if it represents viable but not cultivable portions of the microbiota.
634 Diversity of bacterial communities, as measured by Chao1 was within the range of those
635 found by others for olive varieties used in this study (De Angelis et al., 2015; Cocolin et al.,
636 2013) or for other table olives in general (de Castro et al., 2018; Maoloni et al., 2022;
637 Medina et al., 2016; Penland et al., 2020).
638 Chao1 values were highly variable between and within different groups and the occurrence
639 of significant correlations with physico-chemical variable, counts or the duration of
640 fermentation are hard to interpret. The decrease of fungal diversity with the duration of
641 fermentation is consistent with the hypothesis that fungal diversity decreases over time
642 because of the dominance of a limited number of species over a much more diverse initial
643 community. The number of studies on fungal communities of table olives is more limited
644 than those on bacteria, and sampling criteria and methods differ. Findings on the effect of
645 fermentation time on diversity of fungal communities are contradictory (López-García et al.,
646 2024; Ruiz-Barba et al., 2023a, 2023b).Kamilari et al. (2023) and Penland et al. (2021)
647 reported Chao1 for Cypriot varieties and Nyons olives in the same order of magnitude of
648 those found in our study. Other studies are hard to compare with ours because of the use of
649 different bioinformatic approaches or indices.
650 The union of the core genera for all types of olives resulted in a set of 149 genera, including
651 several genera of LAB, HALAB (which together make up 58% of the core) and Gram-positive
652 and -negative halophiles, but also *Pseudomonadota*. Most of these genera have been found
653 to be abundant in one or more metataxonomic studies in table olives (see (Tsoungos et al.,
654 2023) for recent reviews).
655 *Lactiplantibacillus*, a genus which includes the species *Lpl. pentosus*, which is considered to
656 be well suited to the brine/table olive ecosystem because of its tolerance to salt and

657 polyphenols (Portilha-Cunha et al., 2020) was among the most important bacterial genera
658 but, in several samples was either missing or outnumbered by others. It is frequently found
659 in Italian table olives (Tofalo et al., 2014) and used as a starter because of its fermentation
660 ability, oleuropein degradation and biopreservation and probiotic potentials (Giavalisco et
661 al., 2024; Zotta et al., 2022) However, *Lpl. pentosus* was by far the most frequently isolated
662 LAB in our study (Supplementary Table 9). Given the fact that only 1-2 colonies were picked
663 for each sample, the disproportionate presence of this species among isolates might indicate
664 either that it is more frequently viable at the end of fermentation or a bias due to the
665 culture medium used. HALAB, like *Alkalibacterium* and *Marinilactibacillus* are abundant and
666 prevalent in alkali treated olives, as confirmed by other researchers (López-García et al.,
667 2022; Lucena-Padrós and Ruiz-Barba, 2016; Portilha-Cunha et al., 2020), but we also found
668 them in naturally fermented olives. *Lacticaseibacillus*, which has also potential for use as a
669 starter and functional culture in table olives (Pino et al., 2019) was also among the dominant
670 genera. Several heterofermentative LAB were often (*Lentilactobacillus*) or occasionally
671 *(Limosilactobacillus, Levilactobacillus, Paucilactobacillus* and *Leuconostoc*) dominant or
672 subdominant, but their importance in spoilage or in promoting the quality of table olives is
673 unclear, although *Levilactobacillus* and *Leuconostoc* have been tested as starters in Greek
674 olives (Chytiri et al., 2020; Vougiouklaki et al., 2024). A high proportion of *Lentilactobacillus*
675 may be of concern, because this genus has been associated with spoilage and, in protein rich
676 environments, is associated with the production of biogenic amines (O'Sullivan et al., 2015;
677 Penland et al., 2021; Suzzi and Gardini, 2003).
678 *Celerinatantimonas*, which is abundant in different green and black varieties of olives (e.g.,
679 black Nyons and Kalamata, green naturally fermented Aloreña de Malaga) (Kazou et al.,
680 2020; Medina et al., 2016; Penland et al., 2020), and may contribute to spoilage (de Castro
681 et al., 2022) was abundant and prevalent in Picholine style olives, turning color and black
682 naturally fermented olives, but to a lower extent in green naturally fermented and alkali
683 treated olive, in which *Halomonas, Vibrio, Salinicola* and *Idiomarina* were prevalent and
684 occasionally abundant. This confirms previous findings (Arroyo-López et al., 2021; Lucena-
685 Padrós et al., 2015; Posada-Izquierdo et al., 2021; Ricciardi et al., 2025b; Ruiz-Barba et al.,
686 2023b; Tsoungos et al., 2023). Some of these genera have been associated with spoilage,
687 and more systematic work is needed to clarify their role in table olive fermentation.
688 Because of their phenolic content, salt concentration, and, for most varieties, of their acidic
689 pH, table olives are a relatively safe product and have been rarely associated with food-
690 borne outbreaks, for example botulism (Cawthorne et al., 2005) and salmonellosis
691 (Donachie et al., 2018). The usual low abundance of pathogenic microorganisms in foods
692 and the low taxonomic resolution of amplicon targeted metagenomics pose challenges to
693 the use of metagenomic approaches in the detection of microorganisms relevant for food
694 safety (Ferrocino et al., 2022; Wang et al., 2025). We confirmed our previous finding of the
695 occurrence of sequences assigned to bacterial genera containing pathogenic microorganisms
696 (Ricciardi et al., 2025b). The occurrence of *Salmonella* at relative abundance close to 0.1% in
697 at least two samples, together with previous reports on outbreaks caused by *Salm. enterica*
698 from olives (Donachie et al., 2018) raises some concerns on the occurrence of this pathogen
699 in olive producing environments.
700 Some fungal genera (*Pichia, Candida, Saccharomyces, Wickerhamomyces, Dekkera*) were
701 highly prevalent (>0.50) in table olives, in agreement with what has been found by many
702 studies (see (Ricciardi et al., 2025b; Tsoungos et al., 2023) for recent reviews), although their
703 abundance was very variable. The occurrence of the first 4 genera is very common and they

are among the dominant yeasts in olives (Ricciardi et al., 2025b; Tsoungos et al., 2023) and some species are included in starter cultures. On the other hand, *Zygototorulaspora* has been found as a subdominant or dominant species only occasionally (Arroyo-López et al., 2016; Mougiou et al., 2023). *Dekkera/Brettanomyces*, a genus which includes species which cause spoilage in alcoholic beverages (Tubia et al., 2018) is usually found at low abundances in olives and brines (de Castro et al., 2018; Medina et al., 2018) but can sometimes become subdominant (Michailidou et al., 2021), as in several varieties of naturally fermented olives in our study.

We revealed a striking variability of the composition of both bacterial and fungal microbiota, even within the same olive variety, suggesting the presence of producer-specific, or even barrel-specific, microbial fingerprint. Bar plots of abundances of dominating genera can only provide a general indication of the composition of the microbiota of a specific olive group or variety. Due to differences in methods, it is hard to compare our results with those of other authors, although combining all data in well annotated databases (Ricciardi et al., 2025b) could be of assistance in this task. Overall, the patterns of genera and species found in our study match well with those found by other authors (see (Ricciardi et al., 2025b; Tsoungos et al., 2023) for recent compilations of data).

Only few metataxonomic studies have used either several samples for the same variety from the same firm or samples for the same variety from several firms (see (Argyri et al., 2020; Arroyo-López et al., 2021; Kazou et al., 2020; Lucena-Padrós and Ruiz-Barba, 2019; Penland et al., 2021, 2020) for examples). Although common patterns may emerge in terms of shared composition at the species or genus level, differences emerge within the same firm or variety and have been attributed, among other things, to biogeographical patterns. In our case, we exclude that this variability is a merely technical issue, because Bray-Curtis distance between technical replicates was very low. The diversity, even for the same variety within the same firm, is well reflected in the ordination plots shown in Figures 7 and 8 and in Supplementary Figures 37-39 and 48-50.

Our dataset included spoiled samples, although sensory evaluation was not carried out. It is well known that spoilage affects the microbiota and, in some cases, differential increases/decreases of selected taxa have been associated with specific types of spoilage (Arroyo-López et al., 2021; Castro et al., 2022, 2018; Penland et al., 2021). In our data set, although, at least for fungi, the microbial communities of spoiled samples were often visually different from those of unspoiled samples (see Figure 8), the proportion of spoiled samples was too low and the spoilage categories too varied to draw solid conclusions or to perform inferential analysis.

Despite the variability, the use of inferential methods allowed us to clarify that combinations of olive ripeness and trade preparation across all varieties and firms, varieties within a given combination of ripeness and trade preparation, and firms within a same variety were all factors which significantly affected the composition of bacterial and fungal communities. Differential abundance analysis was harder to interpret but some patterns clearly emerged, like the significantly higher abundance of HALAB in alkali treated olives compared to all other groups, and of a diverse group of *Lactobacillaceae* and of several *Proteobacteria* in naturally fermented olives. The use of inferential methods in the analysis of microbial communities of table olives is comparatively rare. Most studies are small and have an observational approach (Ricciardi et al., 2025b). Comparisons between started and non-started batches, different processes and ripening stages within the same variety (López-García et al., 2022; Penland et al., 2020; Ruiz-Barba et al., 2023b), different locations (López-

García et al., 2022; Lucena-Padrós and Ruiz-Barba, 2019) or between spoiled or unspoiled batches for the same variety (Penland et al., 2021) are more common as are studies on the geographical origin, while, to our knowledge, nobody has ever undertaken a large survey encompassing as many different types and varieties of olives as in our study. (Kamilari et al., 2023) identified differentially abundant genera in three Cypriot varieties; in their case, however, olive variety was confounded with ripeness and trade preparation. Recently (López-García et al., 2025) applied a machine learning approach on metataxonomic data from several studies to evaluate the effect of several variables on bacterial communities of table olives, and the findings on olive processing identify some HALAB as the most important in discriminating alkali treated from naturally fermented olives. Finally, several authors have proposed that metataxonomic analysis of microbial communities may assist in identifying the geographical origin of table olives (Argyri et al., 2020; Ferrocino et al., 2025; Kamilari et al., 2023; Kazou et al., 2020) thus reinforcing an idea of *terroir,* a concept which has originally developed for wine (Belda et al., 2017) and the existence of measurable biogeographical patterns for table olives. This would be consistent with the idea that both dispersal and selection play a role in shaping the microbial communities and thus the organoleptic properties of table olives, which is reinforced by our data. However, while there is sufficient proof that chemometric approaches can assist in the authentication of table olives (Kazou et al., 2025), according to our findings, when the technology is not particularly selective Italian PDO varieties (Bella di Daunia, Nocellara del Belíce and especially, Oliva di Gaeta) can hardly be discriminated from other similar varieties based on microbiota composition. Better integration of metataxonomic data in large metastudies (López-García et al., 2025; Ricciardi et al., 2025b) and integration with metabolomic data may improve this discrimination (Beteinakis et al., 2024).

5. Conclusions

We have presented the largest survey, to date, on the composition of microbial communities of table olives. It is also the first paper providing information on the structure of the microbial communities of Itrana olives, one of the most prominent table olive varieties in Italy. It fully reveals the diversity of bacterial and fungal taxa which may occur in a variety of products over a wide range in geographical origin and technology. We feel that our data confirm that, although selective factors (olive ripeness variety and technological factors, like alkali treatment, salting etc.) play a role in shaping the microbiota, dispersal limitation and insufficient control of key ecological factors (temperature, duration of fermentation, salt) result in a striking variability. This is especially evident for lactic acid bacteria, whose diversity goes well beyond the widespread occurrence of species, like *Lpl. pentosus,* which are traditionally used as starters. Apparently, a wider variety of LAB and HALAB are able to colonize and thrive in this ecological niche, although some seem specifically adapted to some trade preparations, compared to yeasts, whose diversity was significantly lower, with a limited set of species dominating most varieties. Differentially abundant genera and species should be used in designed experiments to clarify to which extent selection, in the absence of dispersal limitation and drift, due to the potential stochastic nature of contamination patterns of barrels used for fermentation within the same firm, governs the establishment of dominant taxa and which is the impact on the safety and sensory quality of table olives. Our data also prove that small surveys (low number of firms and samples) may provide a skewed and limited view even when a study focuses on a single variety. Even the selection of biomarkers for the evaluation of authenticity of PDO varieties may prove tricky: we were

able to detect significant differences between non-PDO and PDO naturally fermented black Itrana olives but the sampling was too unbalanced to warrant the use of machine learning approaches. Better control of contamination and of production technologies may allow a better differentiation among olive varieties.

Data availability.
Sequence data have been deposited in NCBI SRA with bioproject accession PRJNA1292811. The accession list of the runs used in this study is available in Supplementary data and figures. The metataxonomic data have been integrated in the OliveFMBN database (https://github.com/ep142/Metaolive/tree/main/olive_FMBN)

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