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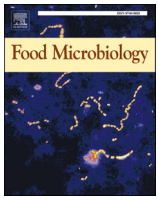

Fungal biodiversity in commercial table olive packages

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ABSTRACT

In this work, a total of 72 commercial table olive packages obtained from different international markets were analysed to determine their fungal biodiversity. Viable fungal counts ranged from the detection threshold (<1.6 $\log_{10}$ CFU/g in 25% of cases) to a maximum of 5.86 $\log_{10}$ CFU/g. Assignation of fungal taxonomy was carried out through a metataxonomic analysis of the ITS region, which revealed that almost half of the total sequences obtained from all packages corresponded to the *Pichia* genus (44.08%), followed by *Citeromyces* (14.45%), *Candida* (8.07%), and *Wickerhamomyces* (6.95%). In lower proportions were also detected other genera such as *Starmerella* (3.60%), *Saccharomyces* (2.24%), *Debaryomyces* (2.08%), and *Dekkera* (2.05%). The statistical analysis allowed to link certain taxa to specific types of elaboration (lye treated, green, and black natural olives), presentation (pitted, whole, or sliced samples), and packaging material/system (glass, PET, plastic bags, and vacuum). Likewise, *Zygotorulaspora* genus was especially sensitive to the presence of potassium sorbate, while other genera such as *Sporobolomyces*, *Moniliella*, and *Gibellulopsis* were more abundant in packages treated with this preservative. Lastly, potential pathogenic fungal genera such as *Alternaria, Kodamaea, Lodderomyces, Malasessia,* or *Aspergillus* were detected in low proportions (<0.3%), although with higher representation in some individual samples. Our results contribute to improving our knowledge of the fungal population associated with this ready-to-eat fermented vegetable, providing us a strong tool to assess the safety, stability, and quality of the final product.

1. Introduction

Tables olives are a fermented vegetable highly appreciated in international markets, with a current worldwide production exceeding 3 million tons per year (IOC, 2021). It is a sector in constant innovation, both in product development and packaging. Thus, we can distinguish a high variety of processing methods (Spanish-style, black or green directly brined olives, Californian style, etc.), presentations (whole, pitted, sliced, etc.), and packaging conditions (glass, PET, tin, or bags, sometimes under vacuum) (Fernández et al., 1997).

Microbial growth control in table olive packaging is not only a key requirement to assure high levels of food safety, but also for the proper preservation and maintenance of product quality. This control can be reached by methods that guarantee the absence of viable microbial cells such as pasteurization or sterilization, or through the preservation of specific physicochemical characteristics (appropriate levels of pH, free acidity, and salt) of the packaging brine, with or without the use of preservatives. In this case, the presence of diverse microbial groups in table olive packaging is common (Fernández et al., 1997). Among the microorganisms that can be found in higher concentrations are those also present during the fermentative process, especially lactic acid bacteria (LAB). However, other undesirable microorganisms such as *Enterobacteriaceae, Vibrio*, *Pseudomonas,* or *Staphylococcus* may appear when storage conditions are not optimal (Benítez-Cabello et al., 2020). Other types of olives presentations, with less repercussion and sold mainly in local markets, are seasoned and stuffed olives. Various studies have reported the seasoning material as a high source of bacteria in the packaging (Benítez-Cabello et al., 2019).

LAB and yeasts can form a mixed biofilm on the olive surface that protects them from environmental conditions allowing them to grow under non-restrictive pH values or low NaCl concentrations (Benítez-Cabello et al., 2020). Thereby, they may remain attached to the surface of the fruit once packaged. Certain yeasts may produce a gradual olive texture degradation through the action of polysaccharolytic

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enzymes which is especially harmful in olive packaging or storage since, it can decrease the shelf-life of the product (Vaughn et al., 1969; Hernández et al., 2007) or produce clouding packaging brines. In this sense, several authors have investigated the use of preservatives to control yeast populations during fermentation and packaging (Bautista-Gallego et al., 2011a,b, 2012; Arroyo-López et al., 2012; Romero-Gil et al., 2016a, 2016b, 2019). However, yeasts also show some desirable activities with important technological and probiotic applications such as lipolytic, β-glucosidase, catalase, and killer activities, providing them a potential use as starters (Hernández et al., 2007; Bevilacqua et al., 2009; Aponte et al., 2010; Romo-Sánchez et al., 2010).

Fungal biodiversity studies in table olive processing are still scarce. Some authors elucidated the fungal composition of black natural table olive fermentations through culture-dependent techniques employing restriction fragment length polymorphism and sequence analyses of the 5·8S internal transcribed spacer and the D1/D2 ribosomal DNA (rDNA) regions of isolates (Nisiotou et al., 2010; Bonatsou et al., 2018). New Generation Sequencing (NGS) and omic technologies have revolutionized the field of food microbiology. In this sense, tools such as amplicon sequencing have been widely used for the identification of bacteria in food and to a lesser extent, in the identification of yeasts and fungi during table olive fermentation (Argyri et al., 2020; Kazou et al., 2020) and the final product (Michailidou et al., 2021). We previously used a metataxonomic analysis to study the fungal population of natural *Aloreña de Málaga* table olives packages (López-García et al., 2021).

In this work, a metataxonomic approach was used to study the fungal population present in commercial table olives packages, to determine the influence of the type of elaboration, presentation style, packaging material/system, and use of preservative. This information lays the foundations for a better understanding of the possible role that these microorganisms play in packaging, in addition to evaluating the hygienic, safety, and quality status of this fermented vegetable.

2. Materials and methods

2.1. Table olive packages

A total of 72 commercial table olive packages were obtained from different supermarkets in Spain (n = 50), Greece (n = 6), France (n = 4), Chile (n = 4), Peru (n = 4), Portugal (n = 2), and Argentina (n = 2), between 2015 and 2017 years. None of them had undergone heat treatment such as pasteurization or sterilization for preservation, which was exclusively based on their physicochemical characteristics (pH, titratable acidity, and salt) or the use of authorized preservatives (potassium sorbate). The samples were analysed in the Food Biotechnology Department of Instituto de la Grasa (CSIC) within the first month of shelf life. They were classified according to the type of elaboration (SS: Spanish style n = 36, GN: green natural n = 26, or BN: black natural n = 10), type of presentation (P: pitted n = 12, W: whole n = 34, or S: sliced n = 26), and packaging material/system (P: PET = 28, B: bag n = 20, G: glass n = 18, or V: vacuum n = 6). They belonged to 16 different olive cultivars (Manzanilla n = 16, Aloreña n = 14, Hojiblanca n = 6, Gordal n = 2, Morona n = 2, Verdial n = 2, Empeltre n = 2, Empeltre Mallorquina n = 4, Kalamata n = 2, Conservolea n = 4, Galega n = 2, Picholine n = 2, Criolla n = 4, Lucques du Languedoc n = 3, Azapa n = 4, and Arauco n = 2). References and origins are shown in Table S1 (supplementary material).

2.2. Physicochemical and microbiological analyses

Table olive packages were analysed within the first month after packaging. The pH of brine was measured using a Titroprocessor model 670 (Metrohm, Switzerland). For the determination of NaCl concentration, 0.5 mL of brine was mixed with 100 mL of distilled water. Then, titration of Cl-was carried out with silver nitrate (AgNO3), using a solution of potassium chromate (K_2CrO_4) as the indicator. The results were expressed as a percentage (w/v) of NaCl.

To determine viable counts of fungi populations on the olive surface (biofilms), 25 g of fruits were washed with sterile saline solution (0.85% v/v) to remove no-adhered cells and pitted at sterile conditions. Then, the fruit flesh (≈22 g) was homogenized in a Stomacher (Seward Laboratory Systems, Inc. Bohemia, NY, USA) for 2 min mixed with 100 mL of sterile saline solution. Afterward, direct or decimal dilutions were plated on YM (yeast-mal-peptone-glucose) agar (Difco TM, Becton and Dickinson Company, Sparks, MD, USA) supplemented with gentamicin sulphate and oxytetracycline as selective agents. Plates were then incubated at 30 °C for 48 h in aerobic conditions. Results were expressed as $\log_{10}$ CFU/g. Then, an ANOVA analysis with Statistica 7.0 software was performed to search for statistical differences between samples ($p \leq 0.05$) grouped according to elaboration, presentation style, and packaging material/system.

2.3. DNA extraction and library preparation

Twenty-five grams of olives from every sample were homogenized with 100 mL of sterile saline solution (0.9% NaCl) in a Stomacher homogenizer for 5 min, and the aqueous phase was centrifuged at 9000×*g* for 15 min. In all cases, the supernatant was withdrawn, and the pellets were washed twice with sterile saline solution before being stored at − 80 °C until use. Total genomic DNA from fruit samples was extracted and purified using the Power Food Microbial DNA Isolation Kit (Mobio, Carlsbad, Calif) according to the manufacturer's instructions, and sent for sequencing to FISABIO (Valencia, Spain). Before sequencing, purified DNA was measured using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, USA), obtaining values above 0.2 ng/μL for all samples.

DNA samples were submitted to PCR amplification of the ITS1 region located between the 18S and 5.8S rRNA genes using designed primers matching the conserved regions, ITS1-F_KYO2 (18S SSU 1733–1753) and ITS2_KYO2 (5.8 2046–2029) (Gardes and Bruns, 1993; Toju et al., 2012). DNA amplicon libraries were generated according to the following PCR protocol: initial denaturation at 95 °C for 3 min, followed by 28 cycles of annealing (95 °C 30 s, 55 °C 30 s, 72 °C 30 s), and a final extension at 72 °C 5 min, using a KAPA HiFi HotStart ReadyMix (KK2602).

Then, Illumina sequencing adaptors and dual-index barcodes (Nextera XT index kit v2, FC-131-2001) were added to the amplicons. Libraries were normalised and pooled before sequencing. The pool containing indexed amplicons was loaded on the MiSeq reagent cartridge v3 (MS-102-3003) spiked with 25% PhiX control to improve base calling during sequencing, as recommended by Illumina. Sequencing was conducted using a paired-end, 2 × 300-pb cycles run on an Illumina MiSeq sequencing platform. Sequencing data have been deposited in the European Nucleotide Archive under the accession number PRJEB50357.

2.4. Bioinformatic analysis

Data obtained from the 72 sequenced samples were analysed using NG-Tax (Ramiro-García et al., 2016) under default parameters. For each sample, only the most abundant sequences (>0.1%) were retained as Amplicon Sequence Variants (ASV); the remaining reads were clustered against those ASVs allowing one mismatch to correct for error sequencing. Taxonomy was assigned using the full UNITE + INSDC (containing 1,796,591 sequences) for the ITS amplicon samples (fungi) (Abarenkov et al., 2020). Alpha diversity and beta diversity indexes were calculated using the R package phyloseq 1.32.0 (McMurdie and Holmes, 2013) and picante 1.8.2 (Wickham, 2016). An ANOVA/Tukey–Kramer (post hoc) test with false discovery rate (FDR) (Benjamini and Hochberg, 1995) was applied to elucidate differences in taxa abundances between the different grouping variables (elaboration, presentation style, packaging material/system, and use of potassium sorbate), considering significant only those differences with a *p*-value

lower than 0.05 and q-value below 0.3 using STAMP 2.1.3 (Parks et al., 2014) software, and Linear Discriminant Analysis (LDA) was developed using LEfse software. Plots were generated using ggplot 2 3.3.2 (Wickham, 2016) and Metacoder 0.3.4 R packages.

3. Results and discussion

3.1. Physicochemical and microbiological analysis

Table olives are ready-to-eat fermented vegetables that need certain levels of pH and salt in the final product to be considered safe. International Olive Council (IOC) regulation (IOC, 2004) establishes a maximum pH value of 4.0 for SS olives when fruits are preserved by refrigeration, by additives addition, modified atmospheres, or by their own chemical characteristics. For natural olives (green or black), the pH value must be less than 4.3. Table S1 shows the physicochemical and microbiological data obtained for the 72 table olive package samples analysed in this work. In the case of pH, this parameter ranged from 2.47 (S146 sample) to 5.40 (S168), with an average value of 3.94 ± 0.63. When grouped samples according to the type of elaboration, the lowest average pH value was found in the SS olives (3.78 ± 0.81), followed by BN (3.96 ± 0.28), and GN olives (4.11 ± 0.33), showing significant differences between GN and SS (Table 1). Albeit all groups were within the limit established by the olive legislation (IOC, 2004), however, some individual samples were out of this limit (Table S1). The pH values obtained agree with those reported by previous studies (López-López et al., 2004), with similar values for SS (3.69 ± 0.03), and directly brined olives (3.92 ± 0.11). No significant differences were neither observed when samples were grouped according to the presentation style. However, in the case of the packaging material/system, the average pH value of B packages was significantly lower (3.34 ± 0.47) compared to P (4.17 ± 0.51), and G (4.23 ± 0.57) (Table 1).

Regarding the salt concentration, olive legislation indicates a lower limit allowed of 4.0% for SS olives preserved by refrigeration or by the addition of preservatives (IOC, 2004). This limit must exceed 5.0 if SS olives are preserved by modified atmospheres or by their own chemical characteristics, or in the case of natural olives, the salts concentration must exceed 6%. In this study values in brine ranged from 3.28% (S139) to 10.59% (S178). When data were grouped according to the type of elaboration, the lowest value was found in SS (5.50 ± 1.13), followed by GN (6.17 ± 1.40) and BN (7.21 ± 1.53), finding significant differences between BN and SS (Table 1). As in the previous case, some individual samples did not meet the established limit (Table S1). López-López et al. (2004) reported lower salt values in brines of packed directly brined olives (4.98% ± 0.22), and similar in SS (5.53% ± 0.17). Regarding the presentation style, NaCl content in W (6.47 ± 1.33) olives was significantly higher than in S samples (5.52 ± 1.33). Lastly, we did not find significant differences between samples grouped according to packaging material/system (Table 1).

Neither of the commercial table olives packaged analysed was subjected to thermal treatment. Thus, viable yeast/moulds may be present during the shelf life. We previously reported viable bacteria in these olive packages reaching a maximum value of 6.73 $\log_{10}$ CFU/g (Benítez-Cabello et al., 2020). In this work, the presence of yeast/mould counts ranged from the detection threshold (<1.63 $\log_{10}$ CFU/g, 25% of cases) to 5.86 $\log_{10}$ CFU/g (S159) (Table S1). The average viable fungi were similar in the three types of elaborations. Thereby, SS showed an average value of 2.87 ± 1.70 $\log_{10}$ CFU/g, followed by GN olives 2.43 ± 1.72 $\log_{10}$ CFU/g, and BN olives 2.12 ± 2.47 $\log_{10}$ CFU/g. No significant differences were found between samples grouped according to the elaboration or presentation style (Table 1). However, regarding the packaging material system, viable fungi were significantly higher in V (4.45 ± 0.74 $\log_{10}$ CFU/g) than in G (2.22 ± 1.80 $\log_{10}$ CFU/g) packages. Glass materials are supposed to have the lowest oxygen diffusion, which could be an explanation to the lower viable fungi detected respecting the Vacuum bag packages, which is expected to have a higher oxygen diffusion. López-López et al. (2004) reported slightly higher viable yeast values in brines from packaged directly brined (average 3.52 $\log_{10}$ CFU/mL) and Spanish style olives (average 3.41 $\log_{10}$ CFU/mL). Other authors reported values higher than 6 $\log_{10}$ CFU/g in fruit samples from commercialised natural olives (Pereira et al., 2008).

Table 1
Physicochemical and microbiological values grouped according to the type of elaboration, presentation style, and packaging material system. Data are expressed as an average value ± standard deviation.

Elaboration style	pH	NaCl (%)	Yeast ($\log_{10}$ CFU/g)
Black natural (BN) (n = 10)	3.96 ± 0.28^{ab}	7.21 ± 1.53^{a}	2.12 ± 2.47^{a}
Green natural (GN) (n = 26)	4.11 ± 0.33^{a}	6.17 ± 1.40^{ab}	2.43 ± 1.72^{a}
Spanish style (SS) (n = 36)	3.78 ± 0.81^{b}	5.50 ± 1.13^{b}	2.87 ± 1.70^{a}
Presentation style			
Whole (W) (n = 34)	3.73 ± 0.66^{a}	6.47 ± 1.33^{a}	2.22 ± 1.91^{a}
Sliced (S) (n = 26)	4.11 ± 0.43^{a}	5.52 ± 1.33^{b}	3.21 ± 1.47^{a}
Pitted (P) (n = 12)	3.74 ± 0.81^{a}	5.55 ± 1.33^{ab}	2.27 ± 2.02^{a}
Packaging material/system			
Plastic Bag (B) (n = 20)	3.34 ± 0.47^{a}	5.77 ± 0.53^{a}	2.31 ± 1.88^{ab}
Vacuum bag (V) (n = 6)	3.94 ± 0.32^{ab}	7.12 ± 0.93^{a}	4.45 ± 0.74^{b}
PET (P) (n = 28)	4.17 ± 0.51^{b}	5.80 ± 1.80^{a}	2.67 ± 1.77^{ab}
Glass (G) (n = 18)	4.23 ± 0.57^{b}	6.11 ± 1.33^{a}	2.22 ± 1.80^{a}

Note: Different superscript letters within the same column, stand for statistically significant differences ($p \leq 0.05$) according to ANOVA analysis.

3.2. Sequencing and quality check

Analysis of eukaryotic amplicon data remains complex in biodiversity studies. In this sense, some metagenomics databases implement their own pipelines to simultaneously host and analyze data sets. However, the pipeline ability to detect eukaryotic DNA relies on comparing the sequence reads to rDNA, ignoring all non-rDNA information (Donovan et al., 2018). This fact added to the incompleteness of the current databases is normally an issue for the data analysis of this kind of amplicons, which led to habitually obtaining a great number of unassigned sequences. Although there are studies that indicate that the use of short sequences is sufficient in diversity studies (Obiol et al., 2020), and is commonly employed to assess eukaryotic diversity in different environments (Saghaï et al., 2015; Pernice et al., 2016; Bahram et al., 2018; Guajardo-Leiva et al., 2018), retrieving a precise taxonomical classification of the amplicon reads (100–250 bp) remains challenging (Lu et al., 2017) as these are still highly dependent on good reference databases for a correct taxonomic assignment (Pedrós-Alió et al., 2018). Besides, although the ITS region has been accepted as the standard barcode marker for fungi, several studies have shown that this region is also not variable for all fungi. Thus, for some *Ascomycota* genera, including *Alternaria, Aspergillus, Cladosporium*, *Penicillium,* or *Fusarium*, identification using the ITS barcode may produce an incorrect taxonomic assignation at the species level (Badotti et al., 2017). Also, databases are more inefficient in this case, providing more non-assigned sequences. Therefore, a study down to the genus level would be a more adequate approximation to obtain robust results when performing a metataxonomic analysis of the ITS region.

In this study, the massive sequencing analysis of the 72 samples generated a total of 1,579,935 raw sequences. After quality filtering, a total of 1,529,779 sequences remained. Three (S104, S139, and S156) of the 72 samples analysed (4.16% of the total) did not pass the quality control, being left out for further bioinformatics analysis. A total of 1258 different ASVs were obtained among all the samples analysed with an average value of 63.20 ASVs per sample. Only 115 ASVs of the total had a representation >0.1% in at least one sample. GN samples showed the

highest number of ASVs, with an average value of 70.6 ± 16.06, followed by BN (61.4 ± 19.34) and SS samples (58.29 ± 16.56). A lower number of fungi ASVs was previously observed in biofilms from natural *Aloreña de Málaga* table olives packages (34.33 ± 2.94) (López-García et al., 2021). Regarding the presentation style, the S samples had the higher number of observed ASVs (74.33 ± 19.21), followed by the P (62.42 ± 17.67), and the W samples (55.39 ± 11.05). Lastly, in samples from the packaging/material system, P packages presented the higher number of ASVs (70.04 ± 18.30), followed by B (64 ± 19.62), G (55.17 ± 10.89), and V (53.4 ± 7.89). A total of 2.29% of sequences were unassigned or unidentified, whilst 1.78% of the total sequences could not reach the genus level, being retained in higher taxonomic levels.

In a previous study, we determined the bacterial diversity of these commercial table olive packages (Benítez-Cabello et al., 2020). A higher number of bacterial ASVs per sample were detected compared to fungi, which was especially evident in Spanish-style samples. Fermentation of olives treated with alkali is mainly carried out by LAB with less predominance of yeasts. On the contrary, in natural olives fermentation yeasts acquire greater importance, as a higher quantity of antimicrobial compounds affecting LAB growth is present (Fleming et al., 1973; Medina et al., 2010).

3.3. Fungal biodiversity

As far as we know, this is the first international study evaluating by molecular methods the fungal biodiversity associated with biofilms obtained from commercial table olive packages. Previous studies have investigated the microbial populations in the cover brines of commercial table olive packages from Portugal, Italy, and Spain using culture-dependent methods (Marsilio and De Angelis, 1993; López-López et al., 2004; Pereira et al., 2008). Only recently, Benítez-Cabello et al. (2020) used a metataxonomic approach to determine the bacterial diversity associated with biofilms obtained from diverse table olive packaging collected around the world. López-García. (2021) also used NGS techniques to study the evolution of the bacterial and fungal diversity during the packaging of traditional *Aloreña de Málaga* table olives. Thereby, few works have focused on the study of yeast/moulds biodiversity associated with olive biofilms, despite its enormous influence on the stability and quality of the final products.

Table S2 shows the different alpha diversity indexes obtained for the 69 table olive packages which passed the quality control. Shannon (H′) index ranged from 0.964 (S125) to 3.745 (S105) with an average value of 2.45, while Simpson index ranged from 0.30 (S125) to 0.947 (S105) with an average value of 0.79, indicating a moderate/high average diversity. H′ index is 0 when the sample contains only one species/genus, and H' is maximum when all species/genus (S) are represented by the same number of individuals. On the other hand, the Simpson index gives greater weight to the abundant species/genus, underestimating the rare species/genus, taking values between '0' (low diversity) up to a maximum of *[1 - 1/S]*. Nearby Shannon (2.11 ± 0.28) and Simpson (0.72 ± 0.09) values were obtained for the fungi population in previous studies (López-García et al., 2021).

Table 2 shows the alpha diversity indexes obtained for the fungal population grouped as a function of the type of elaboration, presentation style, and packaging material system. A good coverage higher than 95% was always obtained, which indicates de quality analysis. No significant differences were observed in the alpha and beta (data not shown) diversity indexes between the samples in none of the groups formed. On the contrary, we observed significant differences in the Phylogenetic diversity (FaithPD) among the samples. FaithPD is a measure of biodiversity which incorporates phylogenetic differences between species/genera. It is defined and calculated as "the sum of the lengths of all those branches that are members of the corresponding minimum spanning path (Faith, 1992), in which 'branch' is a segment of a cladogram, and the minimum spanning path is the minimum distance between the two nodes. A study showed that while phylogenetic and species/genus diversity are very strongly correlated (R^2 = 0.77 and 0.96, respectively), using phylogenetic diversity led to the selection of different conservation priorities than using species richness. It also demonstrated that PD led to greater preservation of 'feature diversity' than species richness alone (Forest et al., 2007).

3.4. Fungal taxonomy

Table S3 shows the different fungi/yeast taxa obtained (with representation >0.1% in at least one sample) for the 69 table olive packages which passed the quality control. The metataxonomic analysis revealed that almost half of the total sequences obtained from all table olive packages corresponded to the *Pichia* genus (44.08%), followed by *Citeromyces* (14.45%), *Candida* (8.07%), *Wickerhanomyces* (6.95%), and other genera in lower proportions, such as *Starmerella* (3.60%), *Saccharomyces* (2.24%), *Debaryomyces* (2.08%), or *Dekkera* (2.05%) (Fig. 1). The rest of the sequences were assigned to ASVs with less than 2% of the total representation.

The detection of these yeast genera in the olive packaging is not uncommon as most of them have been previously reported as dominant during table olives fermentations (Arroyo-López et al., 2008a,b). Bautista-Gallego et al. (2011a,b) reported *Candida diddensiae, Saccharomyces cerevisiae,* and *Pichia membranifaciens* as the most abundant yeast species isolated in green natural olives, and *Debaryomyces etchellsii, C. tropicalis,* and *P. galeiformis* in Spanish style. On the other hand, *Citeromyces* was described as one of the predominant genera during the fermentation of natural green (Alves et al., 2012; Arroyo-López et al., 2016), and black table olives (Porru et al., 2018; Penland et al., 2020). We previously detected sequences from *Candida, Citeromyces, Wickerhanomyces,* and *Debaryomyces* in a high proportion in fruits from *Aloreña de Málaga* table olives packages (López-García et al., 2021). Analysis through NGS techniques to elucidate the fungal community

Table 2
Alpha diversity indexes obtained after grouping table olive packages according to the type of elaboration, presentation style, and packaging material/system.

Elaboration	Observed	FaithPD	Shannon	Simpson	Norm-Shannon	Clonality
Spanish style (SS) (n = 36)	58.29^{a}	2.40^{a}	2.38^{a}	0.77^{a}	0.04^{a}	0.96^{a}
Green natural (GN) (n = 26)	70.60^{a}	3.78^{b}	2.59^{a}	0.81^{a}	0.04^{a}	0.96^{a}
Black natural (BN) (n = 10)	61.40^{a}	2.12^{a}	2.34^{a}	0.78^{a}	0.04^{a}	0.96^{a}
Presentation						
Whole (W) (n = 34)	55.39^{a}	2.31^{a}	2.34^{a}	0.77^{a}	0.04^{a}	0.96^{a}
Sliced (S) (n = 26)	74.33^{a}	3.80^{b}	2.60^{a}	0.80^{a}	0.04^{a}	0.96^{a}
Pitted (P) (n = 12)	62.42^{a}	2.48^{ab}	2.46^{a}	0.79^{a}	0.04^{a}	0.96^{a}
Packaging material/system						
Plastic Bag (B) (n = 20)	64.00^{a}	2.73^{a}	2.42^{a}	0.76^{a}	0.04^{a}	0.96^{a}
Vacuum bag (V) (n = 6)	53.40^{a}	2.13^{a}	2.50^{a}	0.83^{a}	0.05^{a}	0.95^{a}
PET (n = 28) (P)	70.04^{a}	3.30b	2.48^{a}	0.79^{a}	0.04^{a}	0.96^{a}
Glass (n = 18) (G)	55.17^{a}	2.58^{a}	2.44^{a}	0.80^{a}	0.05^{a}	0.96^{a}

Note: Different superscript letters within the same column, stand for statistically significant differences ($p \leq 0.05$) according to ANOVA analysis.

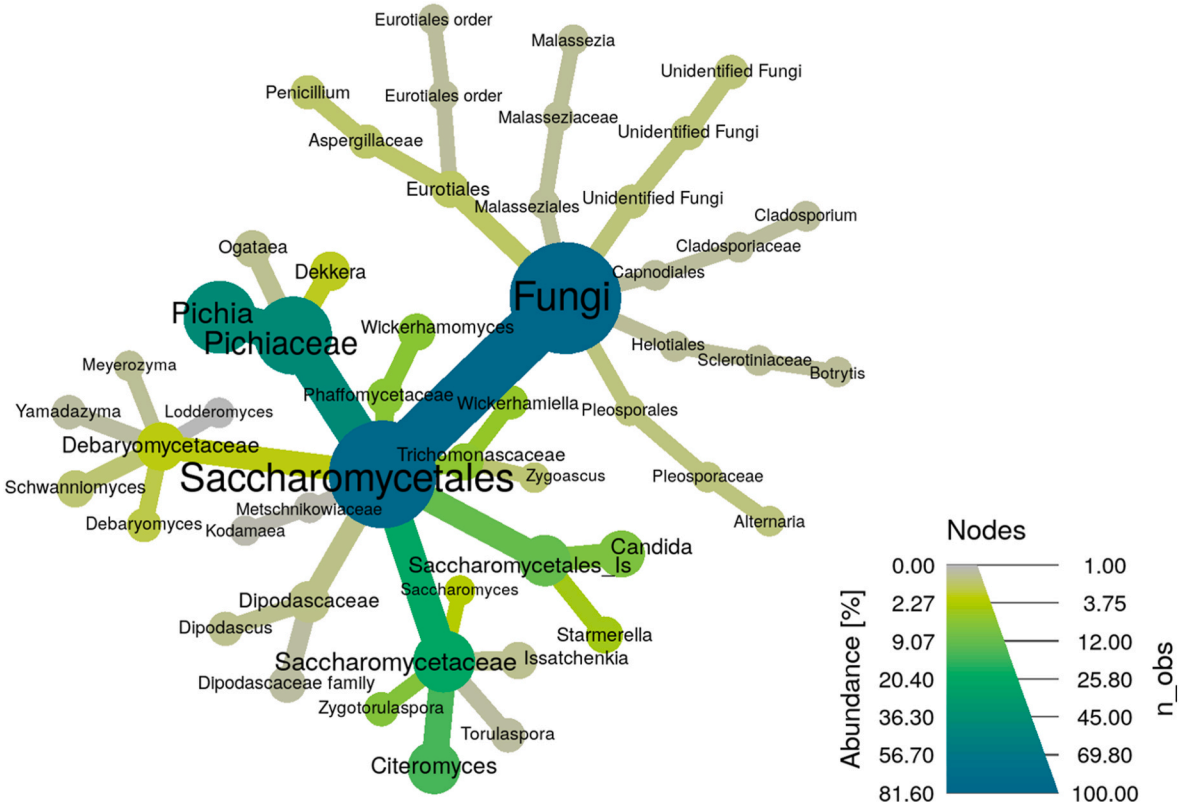

Fig. 1. Heat tree from order to genus level obtained by internal transcribed spacer (ITS) region using metataxonomic data of the 69 table olive packages which passed quality control. The colour and size of the branch are indicative of the abundance of sequences (%). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

during the table olive fermentation has been previously reported in several studies (Arroyo-López et al., 2016; Medina et al., 2018; Demirci et al., 2021). However, few studies have been focused on packaged products.

In this study, we also detected sequences from different genera which contain fungi species with potential pathogenic activity (Table S3). However, there were always detected with a total average value < 0.3%. Thus, genera as *Alternaria* (0.29%), *Kodamaea* (0.19%), *Lodderomyces* (0.14%), *Malasessia* (0.14%), or *Aspergillus* (0.13%) were found. Others such as *Fusarium* (0.08%), *Criptococcus* (0.04%), *Moniliella* (0.03%), *Acremonium* (0.02%) or *Wallemia* (0.01%) were also detected in a very low proportion. However, when we analysed the olive packages individually, *Alternaria,* a genus known for the production of potent allergen compounds (Breitenbach et al., 2002) was present in relatively high proportion in the samples S126 (6.46%) and S127 (6.89%). Besides, these samples had a relatively high proportion of *Fusarium*, with 1.32% and 2.36% of sequences, respectively. *Fusarium* genus groups a large number of species, most of them are parasites of plants, although there are also species capable of infecting humans and animals (Ma et al., 2013). *Kodamaea* genus was present in a relative high proportion in S150 (2.78%), and S151 (5.67%) samples. *Kodamaea ohmeri* species is recognised as a rare pathogen that causes life-threatening infections in humans (Ioannou and Papakitsou, 2020). However, this genus also contains other non-pathogenic species. The sample S105 had a high abundance of both *Lodderomyces* (6.55%) and *Aspergillus* (3.57%). The first one, whose type species *Lodderomyces elongisporus* is considered as a sexual state of *Candida parapsilosis,* and the second one, which is an opportunistic fungus that causes problems mainly in immunocompromised people (Gletsou et al., 2018). This sample was previously characterized by an outstanding presence of *Enterobacteriaceae* and *Pseudomonas* (Benítez-Cabello et al., 2020). Finally, the S176 sample had a high proportion of sequences (4.25%) of *Malassezia* genus. *Malassezia* species are part of the normal skin microbiota, especially those areas rich in fat, but they can also cause more or less important dermal infections (Saunte et al., 2020) and nosocomial outbreaks have also occasionally been described in neonates with intravenous lipid feeding. This lipid-dependency could explain their presence in table olives. Some fungal genera are known for their capacity to produce mycotoxins, chemical compounds produced as a consequence of secondary metabolism. The more important toxins are described in species from *Aspergillus*, *Fusarium*, and *Penicillium* genera (Adeyeye, 2016), all of them detected in our analysis. Their production rate depends on the temperature. In general, this is maximum between 24 °C and 28 °C (optimal yeast growth temperature), so in refrigeration, not only the fungal growth would be lower, but also the proportional production of mycotoxins (Liu et al., 2020). Alsuhaibani (2018) reported a lowering of aflatoxin production and yeast count in nuts stored at refrigeration temperature (4 °C). The legislation establishes that the final product must be free of any microorganisms that could cause a risk for the consumers (IOC, 2004). However, we also have to take into account that DNA studies do not confirm the viability or activity of the taxa found, in addition to their low relative abundance. Further studies are necessary to understand the role of these potential pathogens in the final product.

3.5. Influence of type of table olive package on fungal taxonomy

Fig. 2 shows the relative abundance (%) of fungal taxa when samples were grouped according to the type of elaboration, presentation style, and packaging material/system (Fig. 2C). As mentioned previously, *Pichia* was the most predominant genus in all the groups. However, ANOVA analysis showed ($p < 0.05$) that BN olives presented a significantly higher proportion of the taxa *Kluyveromyces* and *Wickerhamiella* than GN and SS olives (Fig. 3A). Regarding the type of presentation style, ANOVA analysis revealed two taxa being discriminant (Fig. 3B),

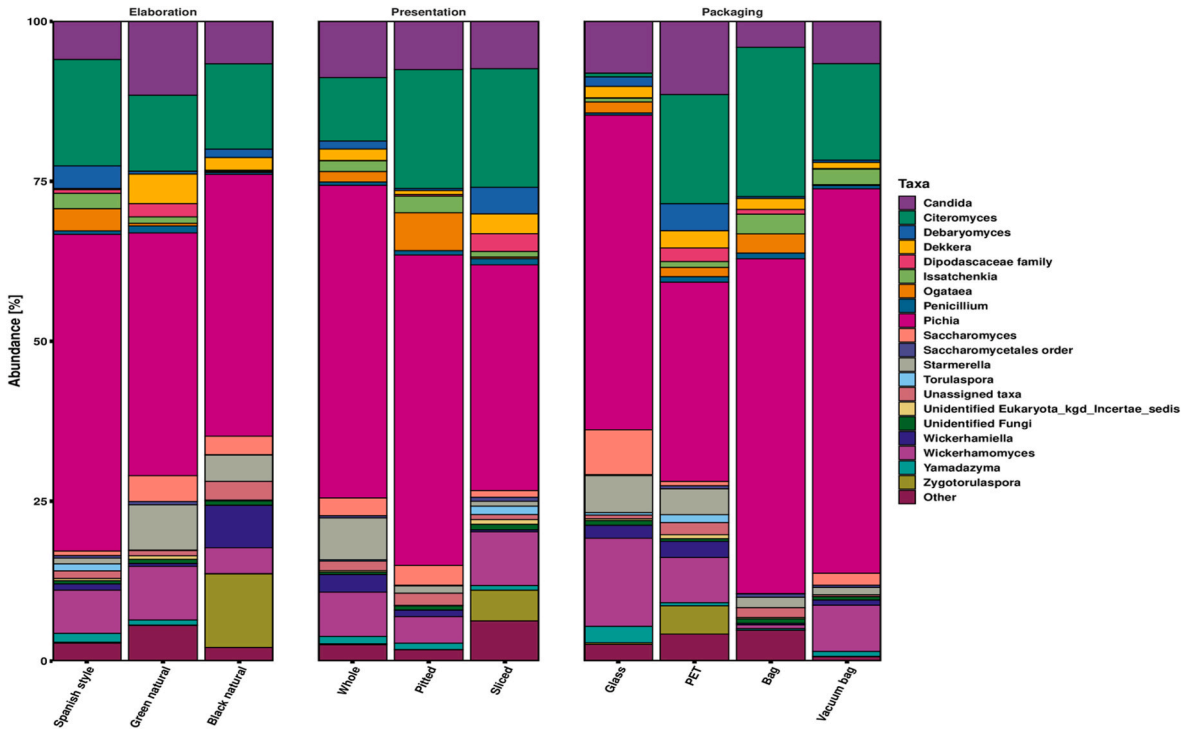

Fig. 2. Relative abundance of fungal taxa (%) obtained by metataxonomic analysis from ITS sequences grouped according to the type of olive elaboration, presentation style, and packaging material/system. Only those taxa with abundance >0.1% in at least one sample are shown.

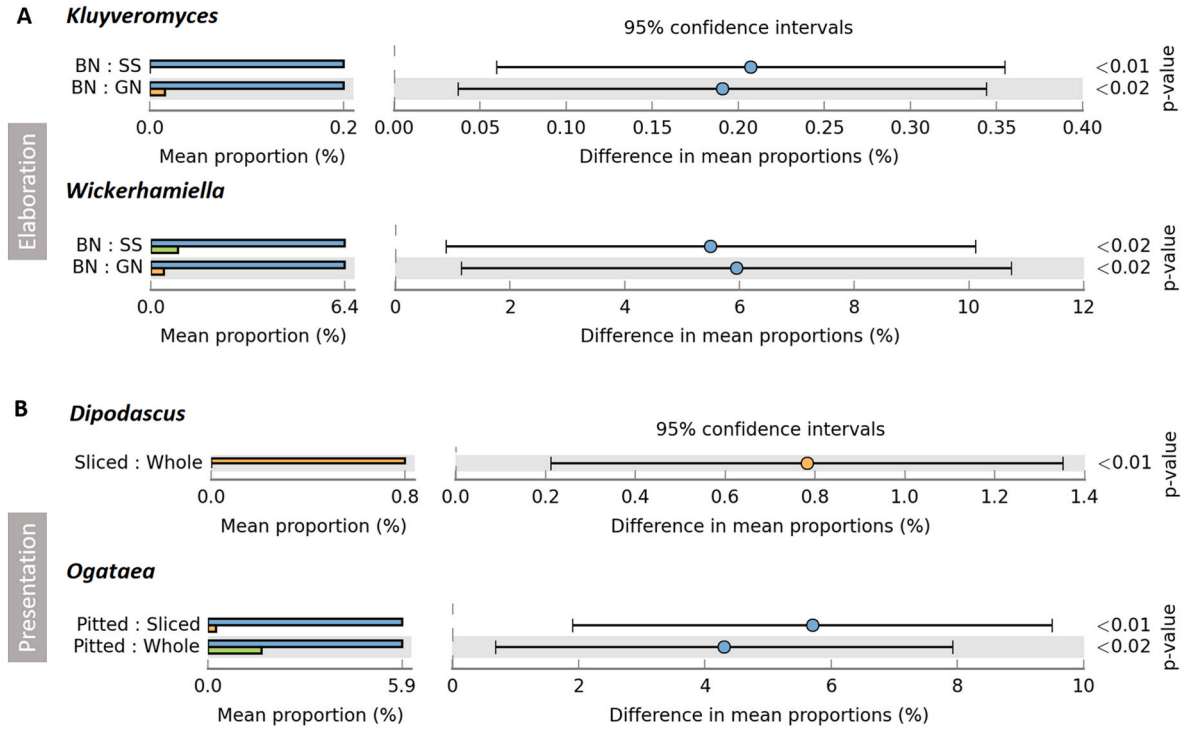

Fig. 3. ANOVA post-hoc comparison showing fungi ASVs with significant differences between pair of samples grouped according to the type of olive elaboration (A) and presentation style (B). Analysis was performed using STAMP and significant differences in taxa were only considered below a p-value of 0.05 and a q-value below 0.3. The mean proportion of sequences within each enterotype, the difference in mean proportions for each pair of enterotypes, and a p-value indicating if the mean proportion is equal for a given pair are illustrated.

Dipodascus genus, which was significantly more abundant in S than in W olives, and *Ogattaea*, more abundant in P olives than S or W.

Linear Discriminant analysis showed *Ogattaea* as a distinctive genus of SS olives, whereas 17 genera were significantly higher in GN (Fig. 4A). *Issatchenkia* was significantly more abundant in P olives, whereas 25 different genera were more abundant in S olives (Fig. 4B).

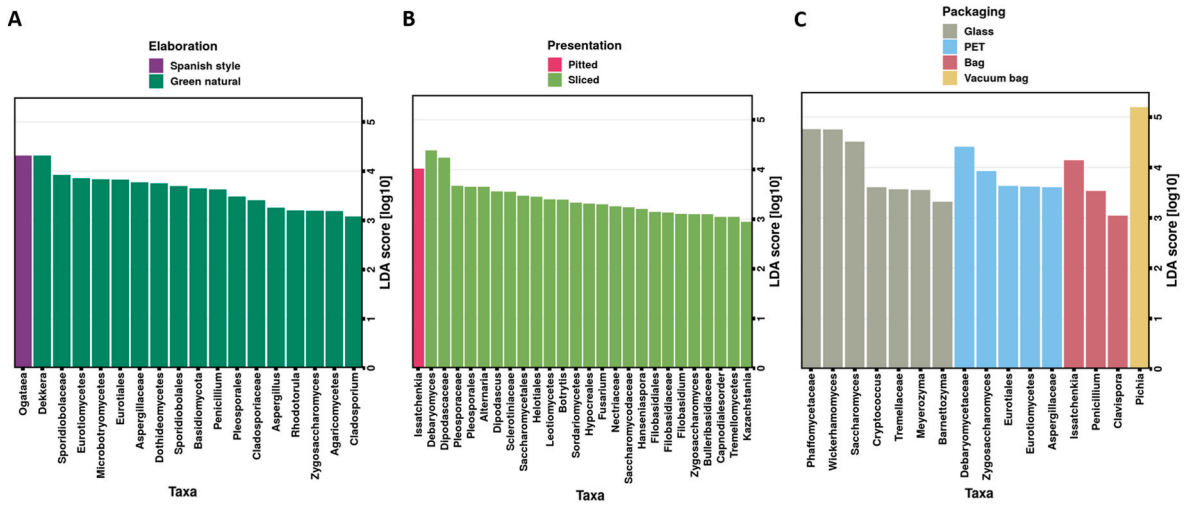

Fig. 4. Linear Discriminant Analysis (LDA) showing differential fungal taxa of each type of olive elaboration (A), presentation style (B), and type of material/system packaging (C). The analysis was carried out with the R package. Only those groups with taxa presenting significant differences are shown.

When the effect of the type of packaging material/system was evaluated, ANOVA analysis did not show significant differences among groups, however, the Linear discriminant analysis revealed that seven taxa (*Phaffomycetaceae, Wickerhanomyces, Saccharomyces, Cryptococcus, Tremellaceae, Meyerozyma,* and *Barnettozyma*) were significantly higher when glass containers were used; five taxa (*Debaryomycetaceae, Zygosaccharomyces, Eurotiales, Eurotiomycetes,* and *Aspergillaceae)* were related to PET containers; three (*Issatchenkia, Penicillium*, and *Clavispora*) to plastic bag containers; while *Pichia* genus was significantly higher in vacuum bags (Fig. 4C). Different factors such as temperature, UV radiation, or oxygen have a direct impact on microbial growth and survival during storage, which ultimately determines the quality of the final product. How these variables affect microorganisms is largely determined by the packaging material/system. We previously found that

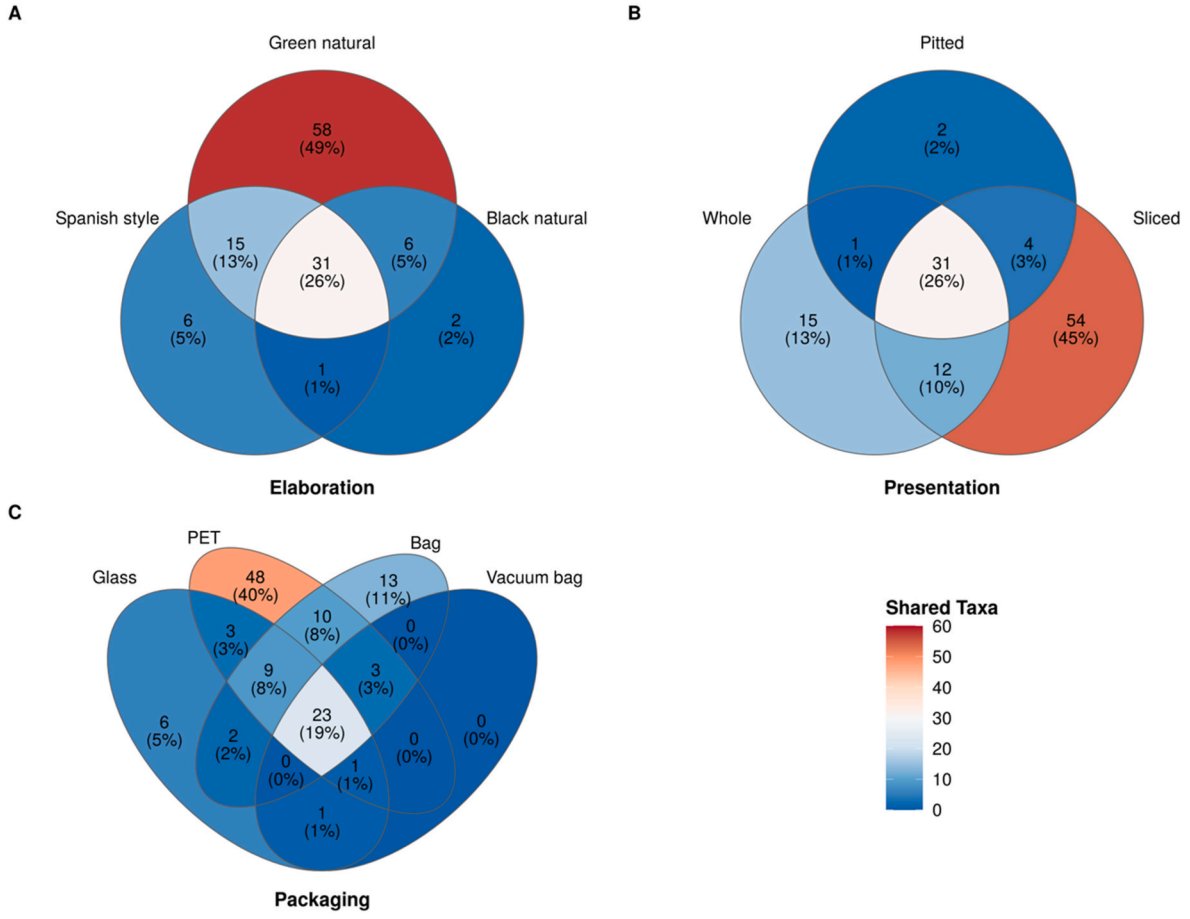

Fig. 5. Venn diagrams showing the total number of unique and shared fungi ASVs grouping table olive packages as a function of the type of elaboration (A), presentation style (B), and packaging material/system (C).

packaging material/system affected the bacterial composition (Benítez-Cabello et al., 2020), possibly associated with the permeability to oxygen of the different materials. Thus, it is expected to have a higher permeability in plastic bags, followed by vacuum bags, PET, and glass containers, as confirmed by a sequential reduction in the abundance of *Pediococcus* genus following this order (Benítez-Cabello et al., 2020). The sensitivity of the different fungal taxa to oxygen may partly determine their development during packaging.

The Venn diagram showed 31 taxa (26%) shared among the three types of olive elaborations (Fig. 5A). Unexpectedly, GN shared more taxa with SS (15) than with BN (6). Furthermore, 58 taxa (49%) were exclusive to GN, 6 taxa (5%) to SS, and 2 (2%) to BN. Regarding the type of presentation style, 31 taxa (26%) were also shared among the three types of samples (Fig. 5B). As expected, more taxa were shared between P and S (4), than between P and W (1). In the same way, we did not expect to find a greater number of taxa (12) shared between W and S than between S and P. Finally, 2 taxa (2%) were exclusive to P, 15 (13%) to W, and 54 (45%) to S samples. Concerning the packaging/material system, 48 (40%) taxa were exclusive from P packages, 13 (11%) from B, and 6 (5%) from G. No exclusive taxa were found in V. Besides, 23 (19%) taxa were shared among all groups of samples. The two groups of samples sharing more taxa (10) were P and B, followed by P and G (3), G and B (2), and G and V (1) (Fig. 5C).

3.6. Influence of potassium sorbate on the fungal taxonomy

Yeasts play an important role during fermentation, enhancing the growth of LAB and releasing a great variety of volatile compounds that provide a distinctive aroma to the final product. However, some of them have polysaccharolytic activity, degrading the cell wall and leading to the softening of the fruit (Arroyo-López et al., 2008a,b), which can be a negative issue during the storage of the product. The effect of different preservatives to control the yeast population during the packaging has been previously investigated (Arroyo-López et al., 2012; Bautista--Gallego et al., 2015; Romero-Gil et al., 2016a,b). One of them is the potassium sorbate, whose effect in combination with other additives and its interaction with pH on the main olive yeast species has already been studied on several occasions (Arroyo-López and Quintana, 2007; López et al., 2007, Arroyo-López et al., 2008a, 2008b). In this study, we evaluated the effect of the application of potassium sorbate in the yeast composition of the packaged. Table S1 shows those table olive packages which were stabilized by the use of potassium sorbate. The ANOVA correlation did not show any significant effect in the yeast population when sorbate was used. However, further LDA analysis elucidated four taxa showing significant differences. Thus, *Sporobolomyces, Moniliella*, and *Gibellulopsis* genera were discriminant when sorbate was employed, being present in higher concentrations after its use. Contrary, *Zygotorulaspora* was especially sensitive to potassium sorbate, which was clearly inhibited by this chemical compound. The increase in the relative abundance of *Sporobolomyces, Moniliella*, and *Gibellulopsis* genera detected in presence of sorbate could be due to both, their insensibility to the preservative, and a clear inhibitory effect in the genus *Zygotorulaspora*.

4. Conclusions

This work has proven the effectiveness of the use of a metataxonomic approach to expand our knowledge of the fungal diversity associated with table olive packaging. The influence that each specific fungal taxon may play on product safety, stability and quality should be confirmed in further studies, albeit the vast majority of the taxa detected in this work have been previously related to the fermentation of this vegetable. Data shows as certain fungal taxa were influenced by the type of olive elaboration, presentation style, packaging material/system, or the use of preservatives.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104082.

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