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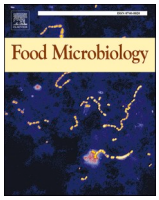

Effect of the cleaning and disinfection methods on the hygienic conditions of fermentation tanks of table olives (*Olea europaea* L.) Negrinha de Freixo cultivar

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ABSTRACT

This study aimed to evaluate and identify the microbial community attached to the surfaces of fermenter tanks used in table olive Negrinha de Freixo cultivar processing through molecular analysis and verify if the cleaning/disinfection was done correctly. Four fermentation tanks previously used in table olive processing were sampled at three different inside areas: upper, middle, and lower. Before sampling, four cleaning/disinfection methods were applied to the tanks, including (*i*) pressurised water; (*ii*) a disinfectant product used to clean bowls (Vasiloxe); (*iii*) 10% sodium hydroxide solution (caustic soda liquid); and (*iv*) a disinfectant product used by the wine industry (Hosbit). For each sample collected, mesophilic aerobic bacteria, yeast and moulds (YMC), lactic acid bacteria (LAB), as well as total coliforms (TC) and *Pseudomonas aeruginosa* were evaluated. The results showed significant differences between the different cleaning/disinfection methods applied. The fermenter sanitised with only pressurised water showed a greater abundance of microorganisms than the others. Mesophilic aerobic bacteria were the predominant population, with counts ranging between 2.63 and 5.56 $\log_{10}$ CFU/100 cm^2, followed by the moulds (3.11–5.03 $\log_{10}$ CFU/100 cm^2) and yeasts (2.42–5.12 $\log_{10}$ CFU/100 cm^2). High diversity of microbial communities was observed between the different fermenter tanks. The most abundant species belonged to *Aureobasidium*, *Bacillaceae, Cladosporium,* and *Rhodotorula* genera. LAB, TC, and *P. aeruginosa* were not detected. This study hopes to improve hygienic conditions and increase the quality assurance and safety of the final product.

1. Introduction

Table olives are among the most popular fermented food products worldwide, especially in the Mediterranean area. There has been a progressive growth in olive production in Portugal, with a value of 23 million tons for the 2021 year harvest (FAOSTAT, 2023). Among the table olives produced in Portugal, Negrinha de Freixo is registered with the Protected Designation of Origin (PDO) status (PDO-PT-0231) (Eambrosia, 2022). Thus, Negrinha de Freixo table olives are a product of great economic and social importance produced in the Trás-os-Montes region, northeast of Portugal (Albuquerque et al., 2019), being necessary to guarantee their food quality and safety. They result from a natural and spontaneous fermentation process conducted by the native microflora present in the fruit skin. The olives are usually placed in 6% salt brine and slowly fermented for 6–9 months. Thus, the olives become edible with high nutritional value, aroma, and flavour characteristics (Malheiro et al., 2014). However, the production process does not always occur as desired, resulting in abnormal fermentations (Lanza, 2013). Potential contaminating microorganisms from different sources, such as polyvinyl chloride (PVC) tanks, fibreglass fermenters, pipes, pumps and water, can alter the fermentation processes (Lee, 2013; Papafotopoulou-Patrinou et al., 2016). Undesirable biochemical

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reactions can occur, producing off-flavours, brine turbidity, and fruit softening (Arroyo-López et al., 2008). One of the biggest causes of contamination in the food industry is the lack of hygiene and cleanliness of the establishments and their equipment, as well as the carelessness of the handlers (Soares et al., 2013). Similarly, at the wine, beer and bread production level, several studies report the presence of undesirable bacteria and yeasts on the surfaces of manufacturing vessels (Loureiro and Malfeito-Ferreira, 2003; Obi, 2018).

The presence of contaminating microorganisms such as faecal coliforms, *Streptococcus*, sulfite-reducing *Clostridium* species, yeasts, moulds and bacteria have been reported in table olives processes. Caggia et al. (2004) isolated and identified *Listeria monocytogenes* in olive samples from traders in Italy. Pereira et al. (2008) verified the presence of faecal coliforms and sulfite-reducing *Clostridium* in packages of table olives sold in the traditional Portuguese market. The microbiological safety of traditional and industrial-processed table olives was also evaluated in different markets in Morocco (Mennane et al., 2021). The results showed a high percentage of olives contaminated with coliforms. Penland et al. (2021), when studying the Nyons table olives that are black table olives with PDO status produced in Southern France, verified that the process environment is critical because it can influence the fermentations, especially the natural ones.

In this context, the establishment of correct hygienic procedures throughout fermentation is necessary to improve the final product's quality and safety. Furthermore, microbiological tests are indispensable in risk management and to ensure the control of microbiological dangers. Thus, the present study aimed to evaluate the microbial community in the surfaces of table olive fermenters from four producers of the PDO Negrinha de Freixo in the Trás-os-Montes region (Portugal) and verify if cleaning and disinfection methods were performed correctly. In this way, it will be possible to guarantee the homogeneity, quality and safety of this excellent product. Thus, the probability of occurring

Fig. 1. Photograph of the table olive fermenters (A, B, C, D) and microbiological surfaces sampling by the swab contact method (E, F).

undesirable operating conditions in the fermentation process will be reduced.

2. Materials and methods

2.1. Sampling

Four table olive producers from Trás-os-Montes region (Mogadouro and Freixo de Espada à Cinta), northeastern Portugal, were selected to evaluate the hygienic conditions of their fermenters. In more detail, the fermenters had the following general characteristics:

- Producer 1: a plastic fermenter, as shown in Fig. 1A is used;
- Producer 2: a cement fermenter, as shown in Fig. 1B is used;
- Producer 3: a fibreglass fermenter, as shown in Fig. 1C is used;
- Producer 4: a plastic fermenter, as shown in Fig. 1D is used.

In each producer, three fermenters were chosen in an aleatory way, being thirty-six samples collected from the surfaces in total. All producers reported that the fermentation conditions were performed according to Negrinha de Freixo table olive PDO requirements (DGADR, 2022). Briefly, the method consisted of washing the fresh olives under running water, placing them directly into tanks, and submerging them in 6% salt brine. Nevertheless, as it was intended to evaluate the microbial community on the surfaces of the fermenters, the sampling was carried out before the start of table olive production, from June to September 2022. Thus, the fermenters were empty and ready to start the production process of table olives for the 2022 harvest.

2.2. Cleaning/disinfection methods applied

Producers were asked about the cleaning/disinfection methods used. It was found that they applied different practices. In general, the samples were collected from tank surfaces considered clean and disinfected, subjected to the following methods: (*i*) pressurised water; (*ii*) a disinfectant product used to clean bowls (Vasiloxe: alkaline detergent based on carbonates and surfactants (<5%), as mentioned in the label); (*iii*) 10% sodium hydroxide solution (caustic soda liquid); and (*iv*) a disinfectant product used by the wine industry (Hosbit: alkaline detergent based on carbonates and sodium, as mentioned in the label).

2.3. Microbiological surfaces sampling - swab contact method

Triplicate samples were collected in different inside areas of the fermenter tanks (upper, middle and lower). A sterile stick swab was dipped into 0.1% sterile peptone water and immediately swabbed in an area of 100 cm^2 (10 cm × 10 cm). The swab was applied with pressure on the surface, with movements from left to right and bottom to top, continuously rotating so that the entire surface of the cotton swab came into contact with the sample (Fig. 1E and F). After swabbing, the stick swab head was gently re-immersed in 0.1% sterile peptone water and mixed by hand. The samples were transported to the laboratory in a refrigerated box and processed within 24 h.

2.4. Microbiological screening criteria

The microbiological criteria followed the guidelines of Instituto Nacional de Saúde Dr Ricardo Jorge (INSA, 2019). The surfaces of the fermenter tanks were considered Zone 1 (Surfaces in contact with ready-to-eat food).

The mesophilic aerobic bacteria count was selected as the total microbiological quality indicator. Yeasts and Moulds (YMC) and Lactic Acid Bacteria (LAB) were selected as environmental and processing quality indicators. Total Coliforms (TC) and *Pseudomonas aeruginosa* were selected as the hygiene indicators.

The culture medium for these microorganisms was performed according to ISO 11133:2004 (ISO, 2014). Plate Count Agar (PCA, HIMEDIA), Violet Red Bile Agar (VRBGA, HIMEDIA), Cetrimide Agar (CA, Liofilchem), Potato Dextrose Agar (PDA, HIMEDIA) and Man Rogosa and Sharpe Agar (MRS, Biolife) mediums were prepared according to the instructions given by the manufacturer.

2.5. Sample processing

Sample processing was performed according to ISO 18593:2018 (Microbiology of the food chain-horizontal methods for surface sampling) (ISO, 2018). The samples were homogenised and serially diluted in 0.1% peptone water up to 10^{-5} dilution. An appropriate volume was inoculated into the specific culture media for each microorganism quantified.

All samples were analysed in triplicate, and the results were expressed as log values of colony-forming units per 100 cm^2 of surface area (log CFU/100 cm^2).

2.6. Enumeration of microbial community

The mesophilic aerobic bacteria counts were obtained by inoculating 0.1 mL of the suspension of the successive dilutions on Petri dishes, containing PCA medium (ISO 4833–1:2013) (ISO, 2013). Each dilution was spread with a sterile spreader. The plates were incubated at 30 °C for 48 h. After this period, all the developed colonies were counted. The YMC were performed on a PDA medium with 0.1% (w/v) chloramphenicol (ISO 21527–1:2008) (ISO, 2008). Seeding was performed by spreading 0.1 mL of each decimal dilution. After incubation at 25 °C for 48 h, the colonies were counted. The TC counts were determined by inoculating 1 mL of each dilution on Petri dishes by the pour-plate technique, using VRBGA medium (ISO 4832:2006) (ISO, 2006). Incubation was carried out at 37 °C for 24 h, followed by the reading of the plates. *P. aeruginosa* colonisation was evaluated by CA medium supplemented with 10 mL of glycerol by spreading 0.1 mL of the respective dilutions. The plates were incubated at 35 °C for 48 h, and subsequently, the plates were read.

The LAB counts were performed by the pour-plate technique, incorporating 1 mL of each dilution in MRS culture medium supplemented with 0.05% (w/v) cycloheximide at pH 5.7 (ISO 15214:1998) (ISO, 1998). The colony count was achieved after incubating the plates at 30 °C for 72 h.

2.7. Identification of microbial isolates

Microbial isolates were first grouped in morphotypes and identified by cultural and morphological characteristics. For each morphotype, two representatives were selected for further molecular identification. Total genomic DNA was extracted using the REDExtract-N-Amp™ Plant PCR kit (Sigma, Poole, UK) following the manufacturer's instructions. The isolates were molecularly identified by ribosomal nuclear DNA (rDNA) amplification and sequencing using the universal primers ITS1F (TCCGTAGGTGAACCTGCGG) and ITS4R (TCCTCCGCTTATTGATATGC) for fungi (White et al., 1990), and the primers 27F (AGAGTTTGATCCTGGCTCAG) and 534R (ATTACCGCGGCTGCTGG) for bacteria (Lane, 1991; Muyzer et al., 1993). The reaction mixture contained the primers (0.4 μL of each at 10 mM), 2 μL PCR buffer (10 mM), 0.4 μL dNTPs (10 mM), 2 μL DNA, 0.1 μL Taq polymerase (5 U/μL) and 14.7 μL of ultrapure H_2O for a final volume of 20 μL. The temperature cycles used in the amplification started with an initial denaturation at 94 °C for 3 min (1 cycle); followed by denaturation at 94 °C for 30 s, primers annealing at 52 °C–56 °C for 50 s, extension at 72 °C for 2 min (35 cycles); and a final extension at 72 °C for 10 min (1 cycle).

The amplified products were purified and sequenced using Macrogen Inc. services (Madrid, Spain). DNA sequences were analysed with DNASTAR v.2.58 software. The isolates were identified by using both National Center for Biotechnology Information (NCBI) and UNITE

databases, executing the BLAST (Basic Local Alignment Search Tool). The obtained results were analysed according to the parameters described by Raja et al. (2017). Operational taxonomic units (OTUs) presenting the lowest E-value and the highest identity score were identified as bacterial, yeast and filamentous fungi species (when identity presented a value ≥ 98%) or genus (when presenting 95%–97% identity). Pure cultures of each identified isolate were conserved and placed in the culture collection of Centro de Investigação de Montanha (CIMO), ESA, Instituto Politécnico de Bragança.

2.8. Data treatment and interpretation

Results are presented as the average of three independent experiments ± standard deviation (SD). Minitab, v. 14 (Minitab Ltd., Coventry, United Kingdom), was used for the statistical treatment of the data. The normality of the data was verified by the Shapiro-Wilk test. Analysis of variance (ANOVA) or ANOVA Welch was carried out to determine if there were significant differences ($p < 0.05$) between samples, depending on the existence or not of homogeneity of variances, respectively. Additionally, if significant differences were detected between treatments, a *post hoc* analysis was performed: Tukey's honestly significant difference test (if variances in the different groups were identical) or Games-Howell test (if they were not). The homogeneity of the variances was tested by Levene's test. The probabilities $p < 0.05$, $p < 0.01$ and $p < 0.001$, were considered significant, very significant and highly significant, respectively. The microbial counts of the surface samples were compared against the guidelines established by Instituto Nacional de Saúde Dr Ricardo Jorge (INSA, 2019).

The abundance and diversity of microbial community detected on the table olive fermenters were measured through abundance, relative abundance (RA, %), and diversity index. RA (%) of a microbial OTU was determined by the total number of isolates of an OTU divided by the total number of isolates of all OUTs. The diversity was estimated by computing Shannon–Wiener (H) index in Species Diversity and Richness v. 4.0 software (Seaby and Henderson, 2007). This index is one of the most used to estimate species diversity in environmental (Mestre et al., 2011) and food (Setati et al., 2012) samples, including table olives (Cocolin et al., 2013) and gives equal weight to rare and abundant species.

A Principal Component Analysis (PCA), an unsupervised multivariate recognition tool, was used to verify how the cleaning/disinfection methods affects the abundance data of the different microorganisms (bacteria, moulds and yeasts). Results are discussed based on 2D or 3D plots of the first two or three principal components (PC) functions, respectively. The open-source statistical program R (version 3.6.2) was used. The following R libraries were used: ggplot2, FactoMineR, factoextra and pca3d.

3. Results and discussion

3.1. Quantification of the microbial community present in surface samples

The mean values of the community of each microbial group determined in the different areas of the fermenter tanks, after applying the cleaning/disinfection methods, are presented in Table 1. It was observed that the mean values of mesophilic aerobic bacteria, moulds and yeasts varied among the producers. The mean values ranged between 2.63 and 5.56 log CFU/100 cm^2 for the mesophilic aerobic bacteria, 3.11 and 5.03 log CFU/100 cm^2 for the moulds, and 2.42 and 5.12 log CFU/100 cm^2 for the yeasts (Table 1). In all analysed samples, there were high values of microbial load. The mesophilic aerobic microorganisms are considered total microbiological quality indicators. They may provide helpful information on the cleaning/disinfection procedures used by food producers. In fact, according to Instituto Nacional de Saúde Dr Ricardo Jorge (INSA, 2019), the fermenters are considered surfaces that come into contact with ready-to-eat food (Zone 1), being the maximum values allowed for mesophilic aerobic bacteria counts less than 2 log CFU/100 cm^2 (INSA, 2019). So, the determined results were considered unsatisfactory and indicated that process failures occurred, requiring a new cleaning/disinfection. Likewise, high yeast and mould counts reveal low environmental and processing quality. However, there is no limit to yeast and mould counts established by Instituto Nacional de Saúde Dr Ricardo Jorge for these surfaces (INSA, 2019). According to the information provided by the producers, the fermenters are normally exposed to air, without a lid, during the campaign break. This situation could explain the high number of microorganisms determined. Moreover, it is well known the ability of fungi to develop under adverse conditions of temperature and humidity showing great tolerance to environmental changes (Talley et al., 2002). Thus, these results suggest that before table olives processing, it is very important to check the hygiene level of the materials in order to avoid future problems.

On the other hand, on any food contact surface, the formation of films into which microorganisms can become adherent may occur. Analyses performed on surfaces that came into contact with the brine during the Spanish-style Gordal cv. green olive fermentation, showed the formation of polymicrobial communities (Domínguez-Manzano et al., 2012). Studies carried out by Grounta et al. (2015) confirmed the ability of microorganisms to adhere to plastic vessel surfaces used in green table olive fermentation and form biofilms, as well as their persistence to applied cleaning treatments. In our study, regarding the cleaning/disinfection method, no significant differences were observed between the application of caustic soda (Producer 3) and Hosbit detergent (Producer 4) when comparing the microbiological groups. Considering both producers (Table 1), the mean values of total count ranged between 2.63 and 2.85 log CFU/100 cm^2 for mesophilic aerobic

Table 1
Mean values of mesophilic aerobic bacteria, moulds and yeasts in different areas of the fermenter tanks (upper, middle and lower) for each table olive producer.

				Inside areas of the fermenter tanks		
		Producers	Mean values	Upper area	Middle area	Lower area
Total count (log_{10} CFU/100 cm^2)	**Aerobic Mesophiles**	Producer 1	5.56 ± 0.16^{C}	$5.63 \pm 0.04^{b,D}$	$5.69 \pm 0.03^{b,C}$	$5.34 \pm 0.04^{a,C}$
		Producer 2	4.73 ± 0.04^{B}	$4.76 \pm 0.01^{a,C}$	$4.73 \pm 0.02^{a,B}$	$4.70 \pm 0.05^{a,B}$
		Producer 3	2.85 ± 0.51^{A}	$3.44 \pm 0.16^{b,B}$	$2.39 \pm 0.38^{a,A}$	$2.72 \pm 0.14^{a,A}$
		Producer 4	2.63 ± 0.32^{A}	$2.91 \pm 0.16^{a,A}$	$2.39 \pm 0.38^{a,A}$	$2.60 \pm 0.19^{a,A}$
	Moulds	Producer 1	5.03 ± 0.16^{C}	$5.13 \pm 0.01^{b,C}$	$5.14 \pm 0.08^{b,C}$	$4.83 \pm 0.10^{a,C}$
		Producer 2	3.11 ± 0.06^{A}	$3.11 \pm 0.06^{a,A}$	$3.08 \pm 0.07^{a,A}$	$3.16 \pm 0.05^{a,A}$
		Producer 3	3.64 ± 0.75^{B}	$4.13 \pm 0.01^{b,B}$	$4.14 \pm 0.08^{b,B}$	$2.65 \pm 0.21^{a,B}$
		Producer 4	$3.25 \pm 0.13^{A,B}$	$3.21 \pm 0.05^{a,A}$	3.31 ± 0.18^{aA}	$3.23 \pm 0.15^{a,B}$
	Yeasts	Producer 1	5.12 ± 0.39^{C}	$4.59 \pm 0.10^{a,D}$	$5.27 \pm 0.04^{b,B}$	$5.51 \pm 0.05^{c,C}$
		Producer 2	3.09 ± 0.14^{B}	$3.00 \pm 0.18^{a,C}$	$3.18 \pm 0.11^{a,A}$	$3.12 \pm 0.09^{a,B}$
		Producer 3	$2.74 \pm 0.56^{A,B}$	$2.16 \pm 0.17^{a,A}$	$3.04 \pm 0.67^{a,A}$	$3.02 \pm 0.11^{a,B}$
		Producer 4	2.42 ± 0.23^{A}	$2.58 \pm 0.13^{a,B}$	$2.26 \pm 0.30^{a,A}$	$2.42 \pm 0.15^{a,A}$

Values are expressed as mean ± standard deviation (n = 9). Values followed by different uppercase letters within a column indicate statistically significant differences ($p < 0.05$) between producers considering the same inside area of the fermenter tanks. Values followed by different lowercase letters within a line indicate statistically significant differences ($p < 0.05$) between the inside areas of the fermenter tanks for the same producer.

bacteria, followed by the moulds (3.25–3.64 log CFU/100 cm^2) and yeasts (2.42–2.74 log CFU/100 cm^2). The products used by these two producers have sodium in their composition (sodium hydroxide and sodium carbonate). Producer 2 also used an alkaline detergent based on carbonates. Possibly, it is also of sodium; however, this fact is not known. Sodium hydroxide and sodium carbonate are known cleaning agents; however, in conjunction, sodium carbonate may inhibit the cleaning ability of NaOH (Atwell et al., 2017). These authors verified that sodium hydroxide >1% would clean well if sodium carbonate was less than 12%. Thus, it is better to use the two compounds separately. Additionally, significant differences ($p < 0.05$) were observed when cleaning/disinfection was performed with pressurised water (Producer 1) or water with the alkaline detergent - Vasiloxe (Producer 2). Thus, the mean values of total count ranged between 4.73 and 5.56 log CFU/100 cm^2 for mesophilic aerobic bacteria, followed by the moulds (3.11–5.03 log_{10} CFU/100 cm^2) and yeasts (3.09–5.12 log CFU/100 cm^2) (Table 1). Among the cleaning methods evaluated in our study, the Hosbit (alkaline detergent based on carbonates and sodium) displayed the best results, being more effective against mesophilic aerobic bacteria and yeasts, with a reduction of up to 2.9 and 2.7 log CFU/100 cm^2 when comparing with the highest value (Producer 1). Nevertheless, the table olive producers must be informed that they must perform a new cleaning/disinfection of the fermenters' surfaces before starting table olive production by natural fermentation.

Regarding the sampling sites – upper, middle, and lower - a similar behaviour was observed between producers, as mentioned before. With rare exceptions (such as, Producer 2 - Moulds), the lowest counts in terms of mesophilic aerobic bacteria, moulds and yeasts were obtained for Producers 3 and 4 at the three sampled points. Considering each producer and comparing the sampled areas, no significant differences were observed between locations in most situations, suggesting that the cleaning/disinfection process was carried out homogeneously (uniformly) across the entire surface. Similar results were observed by Grounta et al. (2015) between three sampling points (top, middle, and bottom) in the fermentation vessels used in green table olive processing. However, in some situations of the present work, differences were observed between the sampling areas, such as: (*i*) Mesophilic aerobic bacteria in Producers 1 and 3 - the lowest values were obtained in the lower (both producers) and middle (Producer 3) areas; (*ii*) Moulds in Producers 1 and 3 - the lowest values were obtained in the lower part; and (*iii*) Yeasts in Producer 1 - the lowest values were obtained in the upper part. As in most cases, the fermenters are not covered with lids, these results suggest that in some situations, the areas most exposed to outside air, such as the upper areas, may have higher counts of microorganisms, mainly mesophilic aerobic bacteria and moulds.

In our study, in the surface samples analysed, the presence of LAB, TC, and *P. aeruginosa* was not detected. This fact suggests the application of good hygiene conditions, indicating, for example, the absence of soil particles that could have adhered to the olives when they were collected, and that could have been deposited on the walls of the fermenters after natural fermentation. If this had happened, TC and *P. aeruginosa* might have been detected. Furthermore, as no LAB was detected, this fact indicates that the surfaces of the fermenters sampled do not maintain an autochthonous flora typical of the fermentation process. The maintenance of flora specific to the fermentation process or that may be harmful to the process happens in the production of wine (Renouf et al., 2006; Guzzon et al., 2017), beer (De Roos et al., 2019) or bread when wooden barrels or kneaders (Elechi et al., 2022) are used. Our results also indicate that using starter cultures in olive production by natural fermentation may be interesting in the future to control fermentations better.

3.2. Identification and characterisation of the microbial community adhered to the surface of fermenters

The microbial community present in the fermenters' surface samples was analysed by amplifying and sequencing the internal transcribed spacer (ITS) region of the rDNA. Across all the fermenters, it was obtained a total of 67 OTUs (Fig. 2A). Thirty-four were identified at Producer 1 and 29 at Producer 2, followed by 26 and 17 OTUs for Producers 3 and 4, respectively (Fig. 2A and B). The relationships between the four cleaning methods and microbial community in surfaces samples, bacterial and fungal taxa (OTUs associated at the genus or species level) that were specific or shared among the different samples were represented using the Venn diagram (Fig. 2A). Regarding the microbial distribution, Producers 1 and 3 had a higher specific number of OTUs ($n = 14$ and 12, respectively). On the other hand, eleven OTUs were shared between Producer 1 and Producer 2, using pressurised water as a cleaning method.

Nonetheless, only one OTU was shared by Producers 1, 3 and 4, and no shared OTUs were identified for Producers 1, 2 and 4. Only four OTUs were found in common when all methods were considered, namely: *Aureobasidium, Bacillaceae bacterium*, *Cladosporium* sp., and *Rhodotorula* sp. Several studies report a high abundance of the genus *Aureobasidium* and *Rhodotorula* species in the endosphere of fresh olives and in the process of natural fermentation (Pereira et al., 2015; Bonatsou et al., 2018; Penland et al., 2020). *Cladosporium* genus can inhabit internal and external environments in mould form, appearing early as endophytic in reproductive organs of different olive tree cultivars (Martins et al., 2021). In addition, their occurrence has recently been identified as spoilage microorganisms in black olive fermentations (Penland et al., 2021). *B. bacterium* belonging to the *Bacillaceae* family, characterised by its robustness and resistance due to the ability to form endospores, is found in the most diverse habitats. The endospores allow the adherence to inert surfaces of food processing equipment (Heyndrickx, 2011). Members of the *Bacillaceae* family have a specific action in the fermentation of foods, such as soybeans and wine, and in more current studies in the manufacture of liqueurs (Tong et al., 2022). Our study showed that these microorganisms were the most resistant to the different cleaning methods, always being detected.

3.3. Diversity and composition of microbial community: Comparison between cleaning/disinfection methods

Analysis of the microbial community present in the fermenters of the four producers revealed a total of 54 species, distributed as follows: 26 bacteria, 16 filamentous fungi, and 12 yeasts (Table 2). The fermenters markedly differed in their percentage of microbial adherence, abundance (N), and diversity (H). Overall, it was found more abundance (106) and species diversity ($H = 2.1$) in the fermenters from Producer 1 than in the others, suggesting that applying only pressurised water is ineffective in reducing the microbial load present on the surfaces of fermenters.

The most abundant species were: *A. pullulans* (0.32%), *Bacillus velezensis* (0.29%), *Cladosporium* sp. (0.28%), *Rhodotorula* sp. (0.23%), *B. bacterium* (0.19%), *Acinetobacter lwoffii* (0.15%) and *R. mucilaginosa* (0.14%) (Fig. 3). The results indicate that when cleaning is carried out with only pressurised water, the microorganisms remain more adherent to the surfaces of the fermenters, becoming more difficult to remove them. When Producer 2 applied pressurised water plus a disinfectant product, a slight decrease in the number of microbial species was observed, which was more pronounced in bacteria and yeasts (Table 2). The most abundant species were: *A. pullulans* (0.33%), *Alternaria alternata* (0.27%), and *Wickerhamomyces anomalus* (0.25%), followed by *Debaryomyces hansenii* and *Pichia membranifaciens* (both 0.17%) and finally *B. bacterium, B. cereus* and *B. subtilis,* with a relative abundance of 0.13%. It must be considered that some species of *Bacillus* genera, namely *B. cereus*, are well-known foodborne pathogens. Even though *Bacillus cereus* was present in low relative abundance, it can pose a threat if it exists in high concentrations in the food processing environment. Thus, when detected in the food processing environment, it is necessary to adopt measures for their control to prevent potential foodborne

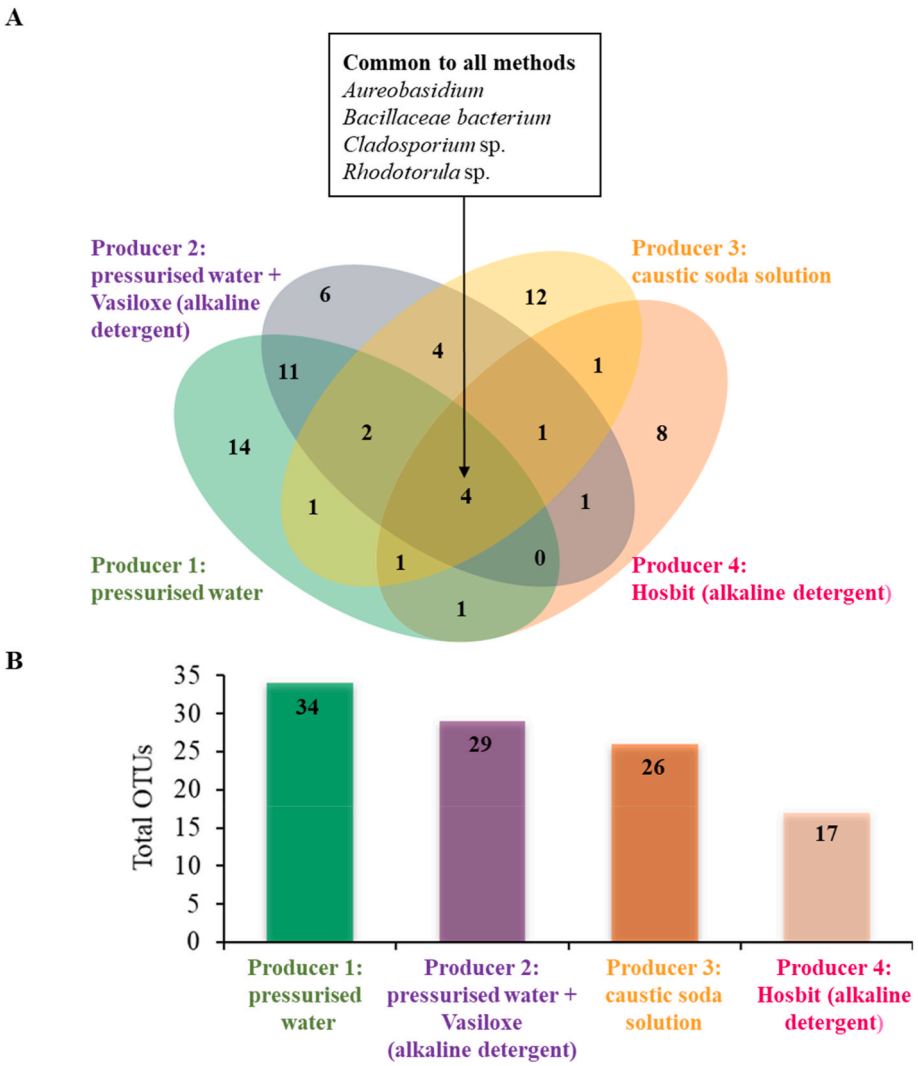

Fig. 2. A) Venn diagram of microbial community distribution among the four table olive producers. Numbers in each circle correspond to the specific or shared number of OTUs (affiliated at the genus or species level) obtained by molecular analysis. Microorganisms common to all methods are highlighted in the box. B) Total of OTUs for each producer.

Table 2
Abundance (N) and diversity of microorganisms detected on the surfaces of the fermenters of four table olive producers. The species diversity index (H) is presented as the total number of each producer.

Parameters	Producer 1	Producer 2	Producer 3	Producer 4	Total
Abundance (N)	106	50	40	26	222
Total nº of species	32	26	16	11	54
Total nº of bacteria	13	10	7	5	26
Total nº of filamentous fungi	9	9	5	4	16
Total nº of yeasts	10	7	4	2	12
Shannon -Wiener index (H)	2.1	1.4	1.1	0.7	1.3

illnesses.

Some species, for example, *Saccharomycetes* sp. (0.14%), *B. paralicheniformis* (0.12%) and *C. globisporum* (0.12%), appeared only in samples collected from Producer 1, while *P. membranifaciens* (0.17%), *A. porri* (0.21%), *B. safensis* (0.06%) and *Corynebacterium variabile* (0.06%) were exclusive of Producer 2. The fact that some species appeared in samples from Producer 1 and disappeared in samples from Producer 2 may be related to the addition of the disinfectant product, which improves the cleaning process. On the other hand, different cleaning treatments may act differently for a particular group of microorganisms. Grounta et al. (2015) verified that cleaning treatments applied to fermentative vessels used in green table olive processing exhibited a higher effect on lactic acid bacteria than on yeasts. A lower number of species were identified in the fermenters from Producers 3 and 4, who used caustic soda solution and a disinfectant used by the wine industry (Hosbit), respectively (Table 2). The microorganisms most frequently isolated were: *Papiliotrema laurentii* (0.50%), *Rhodotorula* sp. (0.50%), *Geotrichum candidum* (0.50%), *C. perangustum* (0.38%), and *Staphylococcus warneri* (0.23%) (Fig. 3). After the application of the Hosbit product, only two yeast species were identified on the surfaces of the fermenters: *Rhodotorula* sp. (0.50%) and *G. candidum* (0.50%). *Rhodotorula* sp. was found in all fermenters, showing to be persistent in the fermenters subjected to all cleaning methods. Recently, yeasts of *Rhodotorula* genera were reported to show endophytic properties, inhabiting the phyllosphere (leaves and twigs) of different olive tree cultivars (Cobrançosa, Galega vulgar, Madural, Picual and Verdeal Transmontana) (Costa et al., 2021). They were also identified in brine and olives of Negrinha de Freixo cultivar (Pereira et al., 2015) and during Greek-style processing of Kalamata natural black olives (Bonatsou et al., 2018), which indicates that the yeasts would come from the fresh fruits. In addition, they have been described as spoilage yeasts

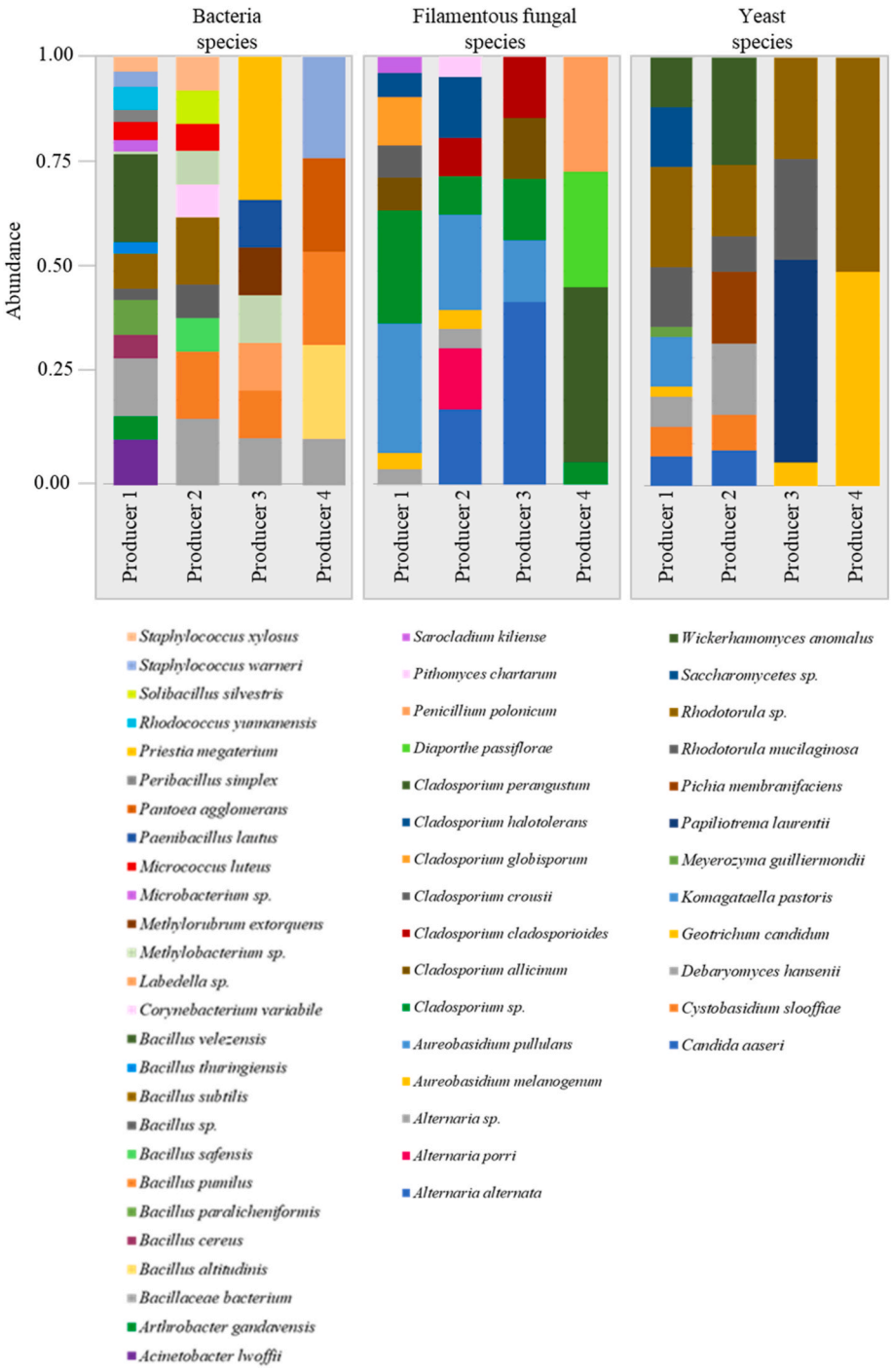

Fig. 3. Relative abundance of the bacterial, filamentous fungal and yeast species identified in the fermenters surfaces cleaned by different methods (Producer 1 - Pressurised water; Producer 2 - Pressurised water + Vasiloxe-alkaline detergent); Producer 3 - Caustic soda solution; Producer 4 - Hosbit-alkaline detergent).

recovered from foods and beverages (Hernández et al., 2018) and from abnormal fermentations in table olives processing (Lanza, 2013). In this sense, the fermenter's hygiene is essential to obtaining high-quality table olives. So, knowledge of the microbiota attached to the fermenter's surfaces and the control of microbial fouling is critical for preventing contamination of the fermentation process.

These results were confirmed when performing a PCA for each type of microorganism (Fig. 4). For bacteria (Fig. 4A), Hosbit (Producer 4) was the most efficient product for reducing bacteria. Regarding the filamentous fungal (Fig. 4B), the four cleaning/disinfection methods were not so efficient against this type of microorganism. On the contrary, the application of caustic soda solution (Producer 3) and Hosbit (Producer 4) reduced the presence of yeast species (Fig. 4C).

Fig. 5 represents a PCA for the four cleaning/disinfection methods and the bacteria, filamentous fungal, and yeast species, where it can be seen that no process was able to eliminate these microorganisms. Overall, 86.57% of the total variance of the data could be explained using three principal factors (PC1 = 37.11%; PC2 = 27.18%, PC3 = 22.28%). Thus, as indicated before, the producers should perform a new hygienization of the fermenters before starting the fermentation process.

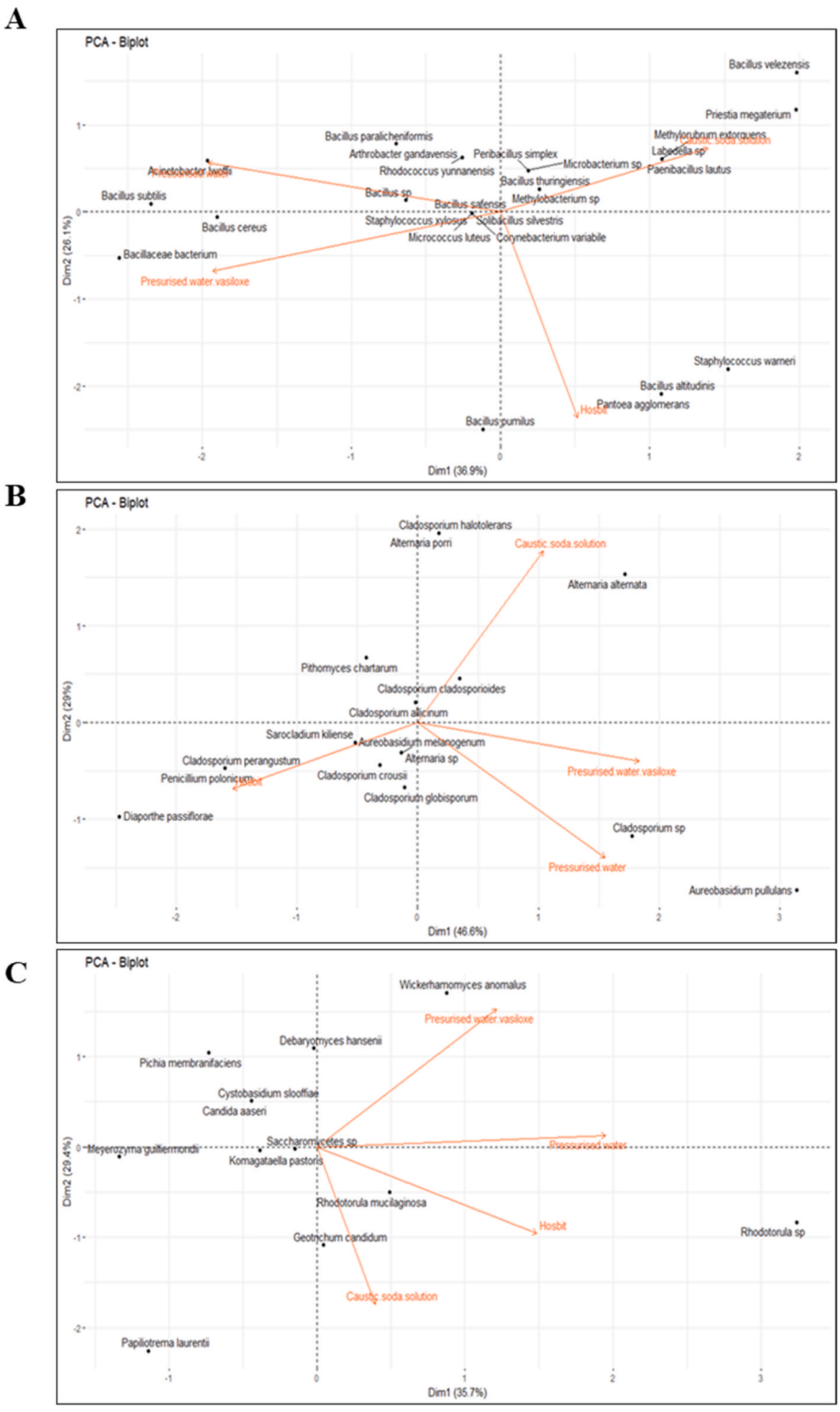

Fig. 4. - Principal component analysis (PCA) applied to cleaning/disinfection methods (Pressurised water; Pressurised water + Vasiloxe-alkaline detergent; Caustic soda solution; Hosbit-alkaline detergent) considering each type of microorganism; (A) bacteria; (B) filamentous fungal and (C) yeast species.

4. Conclusion

This study described for the first time the microbiota that remained adhered on the surfaces of fermentation tanks of table olives Negrinha de Freixo cv. after their cleaning/disinfection. The bacteria most frequently isolated were: *B. bacterium*, *B. cereus* and *B. subtilis*. Concerning filamentous fungus and yeast identification, it was found that *A. alternata*, *Cladosporium* sp., *A. pullulans*; *D. hansenii*, *P. membranifaciens*, and *Rhodotorula* sp. were the most abundant species recovered from the fermenter's surfaces. These microorganisms are common members of the environmental microbiota of fermentation processes and are associated with communities that inhabit the inside of the fruits. As TC and *P. aeruginosa* were not detected, the results obtained in the present work were not worrying. Still, they are an alert to check

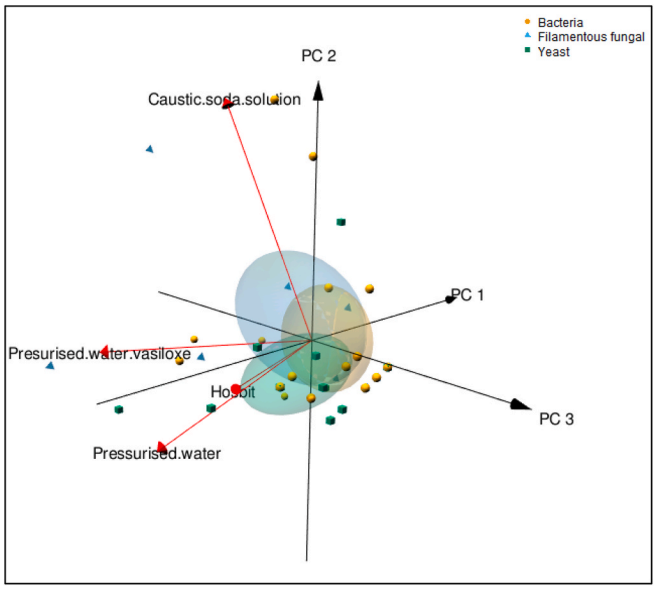

Fig. 5. - PCA obtained from the four cleaning/disinfection methods and the bacteria, filamentous fungal, and yeast species.

the existing hygiene conditions on the fermenter's surfaces before table olives production. In fact, in the artisanal production of this product, the producers must know the fundamental importance of proceeding correctly with the hygiene of the utensils and containers used during table olive production for this to occur adequately. Our study also showed that the cleaning/disinfection processes that proved to be more effective in eliminating microorganisms and preventing the formation of future biofilms were the application of caustic soda solution and the disinfectant used in the wine industry (ex. Hosbit).

Credit author statement

Fátima Martins: Performed the experiments, analysed data, and contributed to manuscript writing. Nuno Rodrigues: Contributed to data analysis. José Alberto Pereira: Contributed to the study design and manuscript editing. Paula Baptista and Elsa Ramalhosa: Contributed to the study design, data analysis, and manuscript writing.

Declaration of competing interest

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

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