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# Research Article
# Isolation of Bacterial Diversity in Oil Mill Water Using Ribosomal Genes Based Fingerprinting from Morocco

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## Abstract

**Background and Objective:** Olive Oil Mill Wastewater (OMW), is a serious problem that threatens both health and the environment, especially aquatic. Bacteria represent an alternative solution to remedy this pollution. To evaluate the possibility of recycling (OMW), 2 olive oil extraction processes (traditional and continuous), taken from Morocco, were studied. **Materials and Methods:** We identified their bacterial diversity and chemical composition. Indeed, we used molecular, using 16S rRNA gene-based fingerprinting of culture-dependent and physico-chemical methods approaches. **Results:** The results of Fourier Transform Infrared Spectrometry (FTIR) show that OMW contains polluting components (especially polyphenols), indicating a difference in chemical composition between traditional and Two-Phase Oil Mill Water (TPOMW) processes. **Conclusion:** Moreover, the molecular study showed a richness of bacteria biodiversity. Therefore, these endogenous bacteria could have a significant role in the purification of olive wastewater.

Key words: OMW, TPOMW, biodegradation, biodiversity, 16S rRNA, bacterial biodiversity, water eutrophication

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The olive oil extraction industry has great economic and social importance in many Mediterranean countries, which produce over 98% of olive oil in the world, including Spain, Italy, Greece and Morocco[1]. Between 1990/91 and 2014/15 world consumption of olive oil increased 1.7-fold and exceeds 2,000,000 t[2].

Despite the importance of this sector, it, unfortunately, creates an alarming environmental degradation through its discharges of OMW, which is composed of water (83-92%), organic matter (4-16%) and minerals (2.1%)[3]. These characteristics cause water pollution of at least 100 fold more than that of urban wastewater[4].

Thus, OMW is a severe and dangerous pollution problem in the Mediterranean countries. They colour the water and their high organic load requires high consumption of oxygen resulting in water eutrophication[5,6]. Morocco is confronting the problem of their elimination from wastewater, which is still flown into the environment without any treatment[2,7].

Although there are several conventional methods for removing OMW, these prove to be less cost-effective, inconvenient for practical use and present many limitations[2]. Alternative methods of elimination OMW based on biological materials have been considered[8-10].

In this study, we have characterized their Bacterial biodiversity and how to remedy the environmental degradation caused by OMW through biotechnological methods.

## MATERIALS AND METHODS

**Study area:** The study was carried out at the Department of Molecular Biology, Applied Microbiology Lab, Spain from October, 2018-January, 2019.

**Sampling:** Samples were taken from Bir Tam Tam location near Fez, Morocco, coordinates: 33°58' 48" North, 4°37′ 48" West. The localization was chosen according to the proximity of water resources (Driss 1 Dam).

**Strains isolation:** For the isolation of cultivable Bacterial populations present in the OMW, 4 solid media were used: Malt agar, Azotobacter medium, universal medium for yeast and medium 220 (German collection of microorganisms and cell cultures, DSMZ)[11]. The colonies were inoculated in the same liquid media and grown at 30°C.

**Molecular methods:** DNA was extracted from OMW using the traditional method of cell lysis, extraction with Phenol-Chloroform-Isoamyl (PCI) and precipitation with isopropanol and sodium acetate.

Cultured bacteria DNA was amplified using 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (TACGGYTACCTTG TTACGACTT)[12].

PCR amplification was performed with 100 µL of the reaction mixture, containing 3 µL of template DNA, 1×PCR Taq polymerase buffer (Promega, Madison, USA), 1.500 mmol $L^{-1}$ $MgCl_2$, 0.100 mmol $L^{-1}$ of each dNTPs (Amersham Biosciences), 2.500 U of Taq DNA polymerase (Promega, Madison, USA) and 0.500 µmol $L^{-1}$ of each primer (Isogen) PCR products were confirmed using 1% of agarose gel electrophoresis. PCR was carried out with a Thermal Cycler AB 2720 (Applied Biosystems, USA) under the following amplification conditions: Initial denaturation 1st cycle at 94°C for 5 min, 35 cycles at 94°C for 45 sec, 49°C for 45 sec, 72°C for 1 min 30 sec and one final cycle at 72°C for 10 min, then holding the temperature at 4°C after the final cycle. The PCR products were purified and sequenced using a BigDye Terminator 3.000 Sequencing Kit (Applied Biosystems, USA) and run on an Applied Biosystems 373S DNA Sequencer.

**Phylogenetic analysis:** The PCRs product was sequenced and compared to various sequences selected from National Center for Biotechnology Information GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). DNA sequence analysis, automatic sample processing and contig editing were performed using DNA baser V3 (http://www.dnabaser.com). Databases and matrices of evolutionary distance were constructed using Clustal X2[13], while the topology, distance and probability of the phylogenetic tree were determined with the MrBayes program[14]. Phylogenetic trees were constructed from evolutionary distances by TreeView software[15].

Nucleotide sequence accession numbers, GenBank and EMBL accession numbers for reference 16S rDNA sequences used in this analysis are in Table 1.

Table 1: Accessions numbers of bacteria sequences submitted to GenBank

| Microorganism | Sequences | Accessions numbers |
|---|---|---|
| Culturable bacteria | 1B | KC707568 |
| | 2B | KC707569 |
| | 10B | KC707570 |
| | 11B | KC707571 |
| | 20B | KC707572 |
| | 31B | KC707573 |
| | 9B | KC707574 |

**Physicochemical and characterization of chemical elements in oil mill water:** Water pH and conductivity were measured in situ using appropriate sensors. The inorganic matter was measured using Total reflection X-Ray Fluorescence (TXRF) and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (UAM Scientific Service, Spain).

Furthermore, Fourier Transformed Infrared spectroscopy (FTIR) was used to analyze the composition of organic matter present in different OMW (UAM Scientific Services, Spain).

## RESULTS AND DISCUSSION

**Physicochemical analysis:** pH and conductivity were measured in two-phase oil mill water (TPOMW from modern oil mill) and traditional OMW. For both samples, the conductivity was 25 and 16 mS $cm^{-1}$, respectively, which greatly exceeds the maximum values of industrial discharges (2.700 mS $cm^{-1}$). The pH of TPOMW and OMW ranged, respectively between 4.860-6.450 and 4.010-5.930[16]. Modern OMW has a pH that goes far beyond the maximum permissible value, while OMW has a very high acidity[17].

Potassium was the most abundant element in the mill's discharge, in TPOMW this concentration was 565 mg $L^{-1}$ (Table 2), which constituted 26% of all chemical elements and in OMW it was 842 mg $L^{-1}$, corresponding to 4.7% of total chemical elements (Table 2).

Besides, iron found in TPOMW and OMW was 6.558 and 8.939 mg $L^{-1}$, respectively (Table 2). These values were higher than the general limit for discharges into surface waters, which is 5 mg $L^{-1}$ [18]. The total phosphorus was much higher in OMW (54.639 mg $L^{-1}$) than in TPOMW (3.355 mg $L^{-1}$) total sulphate was also above these norms, with values of 33 and 36 mg $L^{-1}$ for TPOMW and OMW, respectively (Table 2).

The traditional mills seemed to produce the most toxic effluent. The presence of heavy metal concentrations and other elements showed that the OMW originating from this process exerted higher toxicity. This high toxicity is probably related to their lower water content that leads to the higher concentration of all components[7].

To explain the difference in chemical composition between OMW and TPOMW, infrared spectroscopy was used, this technique is a workhorse for materials analysis in the last years. FTIR spectroscopy has been used by various authors to determine organic matter composition[19,20].

This technique detects the presence of fat, sugars, nitrogenous substances, organic acids, polyols, proteins and polyphenols in OMW (Fig. 1 and Table 3).

According to this analysis, we deduce that modern OMW has a higher content of aromatic compounds (particularly polyphenols), alcohols and organic acids, whereas traditional OMW contains more fatty acids, the band a 1740 $cm^{-1}$, observed is typical stretching C = O band of esters, present particularly in lipids, there is also waxes and other aliphatic structures (Table 3). The measured FTIR spectra show also that the region between 800 and 1400 $cm^{-1}$ is very difficult to analyze because of an overlap of peaks in this region (Fig. 1).

The chemical composition of olives, which is the raw material for olive oil extraction, is very variable and depends on factors such as the olive variety, soil type and climatic conditions[21]. As a result, we have found a difference in chemical composition between traditional and modern OMW.

Many studies have been done on Mediterranean OMW, Fadil *et al.*[22] showed that a high concentration of OMW hurt the health and the environment[22]. It was due to the presence

Table 2: Analysis by x-ray fluorescence reflection from OMW

| | OMW modern | | OMW traditional | |
|---|---|---|---|---|
| Elements | Conc. (mg $L^{-1}$) ± σ | % | Conc. (mg $L^{-1}$) ± σ | % |
| P | <3. 355 | - | 54.639±1.828 | 0.304 |
| S | 32.966±0.783 | 0.564 | 36.581±0.877 | 0.204 |
| K | 565.783±3.183 | 26.766 | 841.515±1.982 | 4.688 |
| Ca | 60.769±0.405 | 1.039 | 82.444±0.449 | 0.459 |
| Ti | 0.108±0.031 | 0.002 | 0.273±0.034 | 0.002 |
| Cr | 0.049±0.020 | 0.001 | 0.119±0.020 | 0.001 |
| Mn | 0.128±0.018 | 0.002 | 0.422±0.021 | 0.002 |
| Fe | 6.558±0.049 | 0.112 | 8.939±0.057 | 0.05 |
| Co | 0.072±0.020 | 0.001 | <0.052 | - |
| Ni | 0.113±0.012 | 0.002 | 0.105±0.011 | 0.001 |
| Cu | 0.245±0.014 | 0.004 | 0.092±0.010 | 0.001 |
| Zn | 0.121±0.017 | 0.002 | 0.568±0.016 | 0.003 |
| Br | 0.478±0.011 | 0.008 | 0.182±0.007 | 0.001 |
| Rb | 0.400±0.011 | 0.007 | 0.497±0.011 | 0.003 |
| Sr | 0.523±0.011 | 0.009 | 0.118±0.118 | 0.001 |

Conc. : Concentration ($cm^{-1}$)

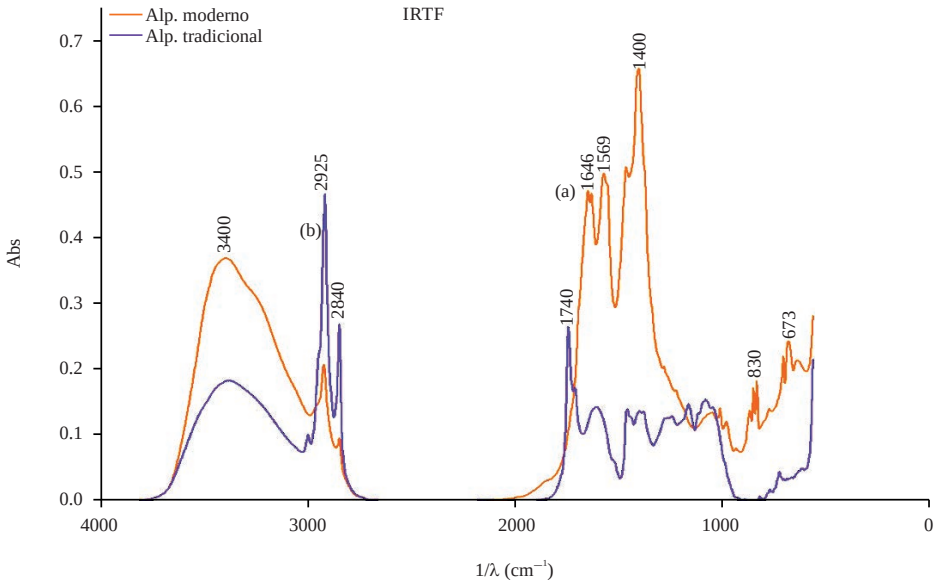


Fig. 1: Fourier transformed infra red spectroscopy spectra of (a) Modern (Alp. Mod) and (b) Traditional (Alp. Trad) OMW

Table 3: Characteristics of FTIR absorption frequencies in the OMW

| 1/λ | Functional groups |
|---|---|
| 3400 ($cm^{-1}$) | Stretching vibrations of O-H (phenols and carboxyl groups) and N-H (amides) |
| 2925 ($cm^{-1}$) | Asymmetric stretching vibrations of C-H corresponding to fatty acids, waxes and other aliphatic structures |
| 2840 ($cm^{-1}$) | Stretching vibrations C = O of esters, ketones and carboxylic groups |
| | Symmetric stretching vibrations of C-H corresponding to fatty acids, waxes and other aliphatic structures |
| 1620-1660 ($cm^{-1}$) | Stretching vibrations C = C of aromatic compounds |
| | Stretching vibrations C = O of amides, primary conjugate acids and or quinones acids |
| 1550-1650 ($cm^{-1}$) | Stretching vibrations C = O of COO- group bending vibrations N-H of amides |
| 1540-1570 ($cm^{-1}$) | |
| 1525 ($cm^{-1}$) | Stretching vibrations C = C of aromatics |
| 1420-1460 ($cm^{-1}$) | Stretching vibrations of C-C aliphatic compounds |
| 1384 ($cm^{-1}$) | Symmetric bending vibrations of $CH_3$ groups |
| 900-1300 ($cm^{-1}$) | Vibrations C-H of deformations of -OH, carboxyl, CO aromatic ethers and NH secondary amides |
| 1040 ($cm^{-1}$) | Silicates, polysaccharides and aromatic ethers |
| 700-900 ($cm^{-1}$) | Aromatic groups |

of toxic compounds in the effluent, such as tannins and simple phenolic compounds, which are responsible for phytotoxic and antimicrobial effects[4,23].

Moreover, Hajjouji *et al.*[24], showed that the biotransformation of olive mill wastewater appears to favour the stabilization of the organic matter through mechanisms analogous to those that lead to the formation of humus in the soil.

The physicochemical parameters and chemical composition measured indicate that OMW may hurt the receiving environment constituted by the Driss I Dam in Morocco.

**Bacterial diversity in olive mill wastes:** Different treatment methods have been developed to eliminate OMW wastewater. These include its direct application in agricultural soils as a fertilizer[6,7], composting methods[25], abiotic[26] or photochemical treatment[27,28]. These methods are expensive or have other inconveniences. Alternatively, a biological system seems to be a suitable approach for such treatment. Consequently, we studied the biodiversity to identify the bacterial communities present in this environment.

We realized by culture-dependent approaches isolation of bacteria from OMW to identify qualitatively the bacterial richness.

In this case, 2 phyla were being noted (Table 4) Firmicutes with the 5 strains (1B, 9B, 10B, 11B and 20B) and Proteobacteria phylum that contains 2 strains (2B and 31B). We subsequently realized a phylogenetic tree corresponding to each phylum (Fig. 2 and 3).

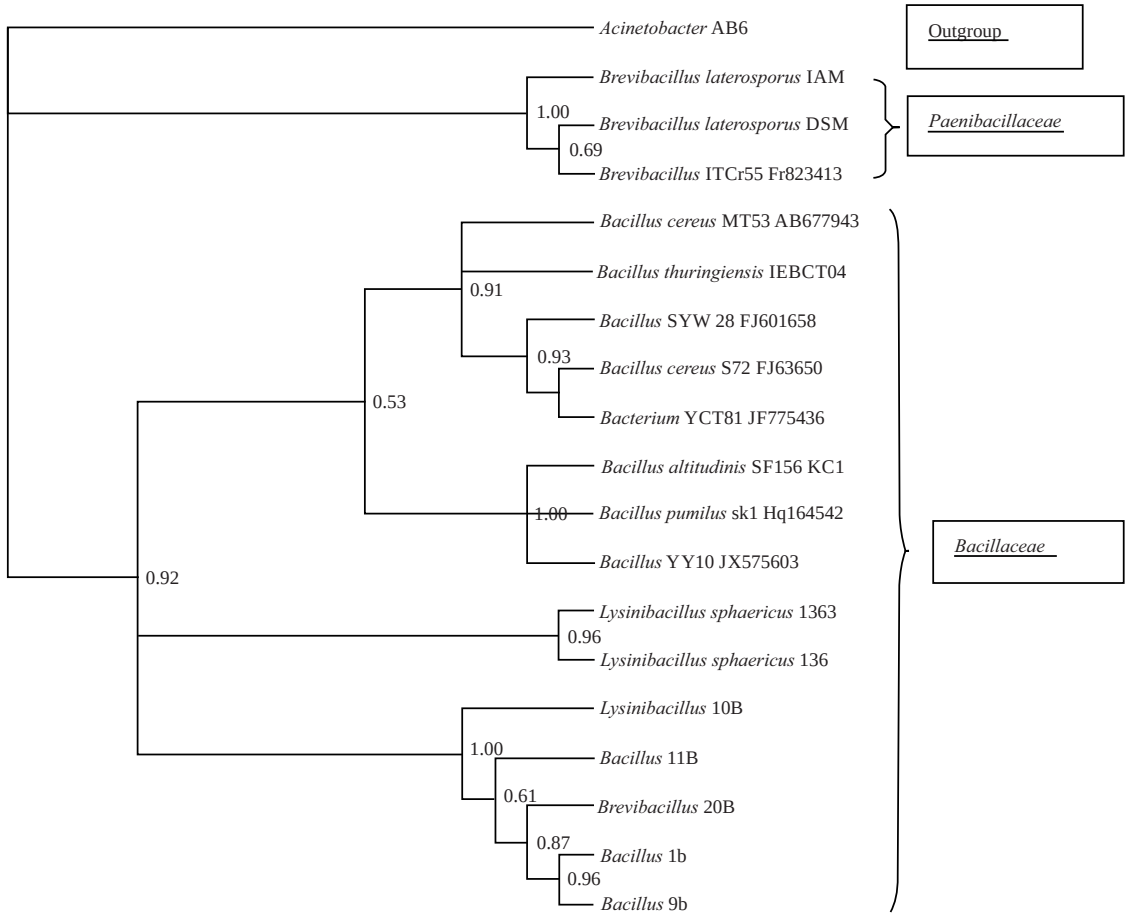


Fig. 2: Phylogenetic tree resulting from comparison of 16S rRNA gene sequences derived from Firmicutes phylum with reference sequences extracted from the GenBank database, using MrBayes program[14]

Moreover, the phylogenetic tree of Firmicutes phylum was illustrated in Fig. 2. They showed that all strains isolated from OMW belong to the family of Bacillaceae and defined an independent cluster within this group. The B20 strain has probably a bioremediation activity because this sequence is closely related (98%) to *Lysinibacillus fusiformis* strain N43 (JQ900515.1), which is a crude oil-degrading bacterial isolate from an ecological region of Assam India (Table 4).

OMW was mostly colonized by bacteria of the Firmicutes phylogenetic group, a similar result was found by Scoma *et al.*[9], which isolates only bacteria belonging to this phylum. In addition, Ntougias *et al.*[16] studied the bacteria presents in olive mill wastes, *Bacillaceae*, *Clostridiaceae*, *Lactobacillaceae* and *Paenibacillaceae*, which are the most abundant taxa within the phylum Firmicutes.

In the event of the phylogenetic tree of *Proteobacteria* phylum were found (Fig. 3), 2B strain isolated from TPOMW was classified with the 31B derived from OMW traditional, these 2 strains appear identical (posterior probability index equal to 1.00) and classified into Gammaproteobacteria with a separate cluster (Fig. 3).

It should be noted that the B2 strain shows a low similarity to *Alcaligenes* sp. BBTR16 (EF471233.1) belongs to the Betaproteobacteria according to the BLAST database but this similarity is weak (91%) (Table 4).

The 31B isolated from traditional OMW which was 98% similitude to *Pseudomonas stutzeri* strain Gr65 (FR667898)[8]. This result seems to conform with the research of Tsiamis[29] which also identifies the same groups.

On the other hand, Vivas *et al.*[30] found that TPOMW was dominated by members of the phylum Proteobacteria, which represents 50% of the total bacterial community existing in OMW[16].

Even though the distribution analysis of the 16S rRNA gene sequences was deposited in NCBI databases by PCR, an amplification survey of 16S rRNA gene databases revealed the presence of 585 deposited sequences of bacteria identified in olive mill waste environments[16].

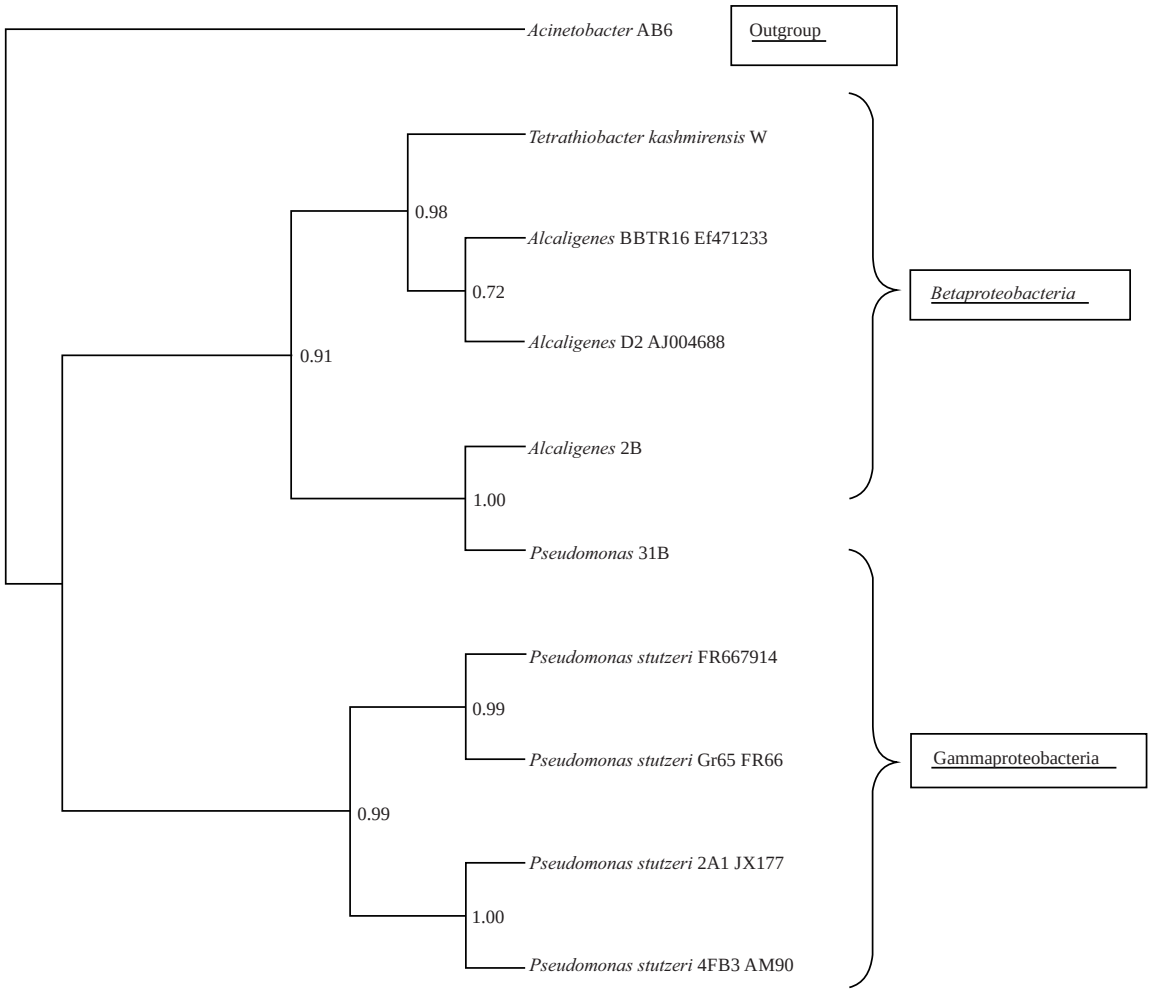


Fig. 3: Phylogenetic tree resulting from comparison of 16S rRNA gene sequences derived from *Proteobacteria* phylum with reference sequences extracted from the GenBank database, using MrBayes program[14]

Numbers at the nodes represent Bayesian posterior probability, the *Acinetobacter* AB1 was used as an outgroup, the GeneBank accession numbers of the sequences are given next to the species name, the average standard deviation of split frequencies = 0.024615 and number of gene ration = 1000000

Furthermore, the endogenous bacteria isolated from OMW and TPOWM could have a significant role in the purification of olive wastewater and reduce the total phenol content[31].

## CONCLUSION

In this study, we have characterized the differences in the chemical composition of different OMW, the modern one has a pH that goes far beyond the maximum permissible value, while the traditional OMW has very high acidity and contains more polyphenol than the modern one. Bacterial diversity was very important in both samples and relatively high in traditional OMW, which indicates that there is a change in Bacterial diversity during the OMW process. These microbes can use in detoxifying processes of the wastewater before rejecting it into nature.

## SIGNIFICANCE STATEMENT

This study discovers, the correlation between the nature of pollution and bacterial biodiversity, these bacterial can use in detoxifying processes of the wastewater before rejecting it into nature. This study will help the researcher to understand the characterization of olive mill wastewater that many researchers were not able to explore.

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