



Evaluation of Established Methods for DNA Extraction and Primer Pairs Targeting 16S rRNA Gene for Bacterial Microbiota Profiling of Olive Xylem Sap

Carmen Haro^{1†}, Manuel Anguita-Maeso^{1†}, Madis Metsis², Juan A. Navas-Cortés¹ and Blanca B. Landa^{1*}

¹ Institute for Sustainable Agriculture, Spanish National Research Council (CSIC), Córdoba, Spain, ² Testsystems LLC, Tallinn, Estonia

OPEN ACCESS

Edited by:

Barbara Pivato,
Institut National de Recherche pour
l'Agriculture, l'Alimentation et
l'Environnement (INRAE), France

Reviewed by:

Jingjing Peng,
China Agricultural University, China
Ben Niu,
Northeast Forestry University, China

*Correspondence:

Blanca B. Landa
blanca.landa@csic.es

[†]These authors have contributed
equally to this work and share first
authorship

Specialty section:

This article was submitted to
Plant Pathogen Interactions,
a section of the journal
Frontiers in Plant Science

Received: 12 December 2020

Accepted: 08 February 2021

Published: 12 March 2021

Citation:

Haro C, Anguita-Maeso M,
Metsis M, Navas-Cortés JA and
Landa BB (2021) Evaluation
of Established Methods for DNA
Extraction and Primer Pairs Targeting
16S rRNA Gene for Bacterial
Microbiota Profiling of Olive Xylem
Sap. *Front. Plant Sci.* 12:640829.
doi: 10.3389/fpls.2021.640829

Next-generation sequencing has revolutionized our ability to investigate the microbiota composition of diverse and complex environments. However, a number of factors can affect the accuracy of microbial community assessment, such as the DNA extraction method, the hypervariable region of 16S rRNA gene targeted, or the PCR primers used for amplification. The aim of this study was to assess the influence of commercially available DNA extraction kits and different primer pairs to provide a non-biased vision of the composition of bacterial communities present in olive xylem sap. For that purpose, branches from “Picual” and “Arbequina” olive cultivars were used for xylem sap extraction using a Scholander chamber device. The DNA extraction protocol significantly affected xylem sap bacterial community assessment. That resulted in significant differences in alpha (Richness) and beta diversity (UniFrac distances) metrics among DNA extraction protocols, with the 12 DNA extraction kits evaluated being clustered in four groups behaving differently. Although the core number of taxa detected by all DNA extraction kits included four phyla, seven classes, 12 orders, 16 or 21 families, and 12 or 14 genera when using the Greengenes or Silva database for taxonomic assignment, respectively, some taxa, particularly those identified at low frequency, were detected by some DNA extraction kits only. The most accurate depiction of a bacterial mock community artificially inoculated on sap samples was generated when using the PowerPlant DNA extraction kit, the combination of 799F/1193R primers amplifying the hypervariable V5–V7 region, and the Silva 132 database for taxonomic assignment. The DESeq2 analysis displayed significant differences among genera abundance between the different PCR primer pairs tested. Thus, *Enterobacter*, *Granulicatella*, *Prevotella*, and *Brevibacterium* presented a significant higher abundance in all PCR protocols when compared with primer pair 799F/1193R, while the opposite was true for *Pseudomonas* and *Pectobacterium*. The methodological approach followed in this study can be useful

to optimize plant-associated microbiome analysis, especially when exploring new plant niches. Some of the DNA extraction kits and PCR primers selected in this study will contribute to better characterize bacterial communities inhabiting the xylem sap of olives or other woody crop species.

Keywords: DNA extraction kits, NGS, 16S rRNA gene, mock community, microbiome, xylem

INTRODUCTION

Olive (*Olea europaea* subsp. *europaea*) is a primary element in the agricultural economy of most countries in the Mediterranean Basin, where about 5 million hectares of olive orchards are grown only in European countries. More than half of the cultivated olives worldwide are in Spain, accounting for 70–75% of world production of olive oil and more than one third for table olives (EUROSTAT)¹. During the last years, the health of the olive groves is being seriously threatened, as a consequence of a notable increase, both in extent and in severity, of diseases caused by diverse pathogens, which are capable of adversely affecting its growth and production. Among olive diseases, those caused by the vascular plant pathogenic bacterium *Xylella fastidiosa* and the soilborne fungus *Verticillium dahliae* are, without a doubt, the two major global threats to olive production worldwide (Jiménez-Díaz et al., 2011; Saponari et al., 2018; Landa et al., 2019; Anguita-Maeso et al., 2020).

Research on plant-associated microorganisms or plant microbiome has gained importance in the last decade as a key component in the health and productivity of the plant (Berg et al., 2014). Thus, recent studies have shown that certain endophytic bacteria are capable of modifying the development of diseases in plants, promoting their growth and protecting them against insects and pathogens (Müller et al., 2015). In addition, they could confer other important benefits for plants, such as greater resistance to stress conditions, alteration in physiological properties, and production of phytohormones and other compounds of biotechnological interest (Porrás-Alfaro and Bayman, 2011; Hacquard and Schadt, 2015; Santoyo et al., 2016). Within the plant tissues, xylem vessels are considered ideal niches for microorganisms by providing an effective internal pathway for distribution throughout the plant and a continuous source of nutrients (McCully, 2001). However, information on the nature and role of the xylem microbiome and its contribution to plant health and crop productivity is still scarce (Anguita-Maeso et al., 2020).

For olive trees, most microbiota studies have focused on determining the microbial composition of its rhizosphere (Mercado-Blanco et al., 2004; Aranda et al., 2011; Berg et al., 2016; Gómez-Lama Cabanás et al., 2018; Lei et al., 2019; Fernández-González et al., 2020) and to which extent these microbial communities can act as potential antagonists of olive pathogens such as *V. dahliae*. Other studies have focused on the effect of abiotic factors (edaphic, climatic, and agronomic) on the olive soil- and rhizosphere-associated

microbiota (Montes-Borrego et al., 2013; Landa et al., 2014; Caliz et al., 2015). More scarce are the works in which the endosphere olive microbiota has been investigated. In those studies, diverse methodological approaches based on next-generation sequencing (NGS) technologies were used to analyze the microbiota composition (Kennedy et al., 2014; Müller et al., 2015; Fausto et al., 2018; Sofó et al., 2019; Anguita-Maeso et al., 2020). Moreover, it has been demonstrated that the method used for DNA extraction can lead to dramatic differences in microbial output composition (Henderson et al., 2013; Brooks et al., 2015), which makes validation of DNA extraction methods with a mock microbial community essential to ensure an accurate representation of the microbial communities in the samples under study.

Most of the referred studies analyzing xylem microbiota have been based on amplicon sequencing using “universal” primers targeting the 16S rRNA gene in bacteria, as it is the most cost-effective and facile tool to provide valuable phylogenetic information for the comparison of bacterial diversity in large numbers of samples. However, the lack of standardization procedures among plant microbiota studies can make the comparison of results among them difficult (Stulberg et al., 2016). Both choice of the hypervariable region of 16S and the primer pair have been shown to influence the description of microbial diversity (Claesson et al., 2010; Fouhy et al., 2015; Teng et al., 2018). Thus, care should be taken in choosing appropriate primer pairs, as limited taxa coverage or overrepresentation or underrepresentation of taxa in a specific environment due to biases in primer amplification could occur that could lead to unreliable results (Claesson et al., 2010; Wasimuddin et al., 2020).

An additional problem when working with plant tissues is the co-amplification of undesirable or non-target sequences from organellar origin (e.g., mitochondria and/or chloroplast DNA) that may represent a major source of “contamination” due to the homology between bacterial 16S rDNA, chloroplast DNA, and mitochondrial DNA. This leads to significant challenges in the selection of appropriate primer pairs to address the study of plant–microbe interactions (Ghyselinck et al., 2013). Several methodologies have been proposed to reduce co-amplification of plant organellar sequences such as a reduction of co-extraction of organellar DNA based on differences in methylation density (Feehery et al., 2013), blocking primers and suicide polymerase endonuclease restriction (SuPER) (Green and Minz, 2005), and the use of specific mismatch primers during PCR amplification (Beckers et al., 2016). Among them, the preferred or most used approach is the use of specific mismatch primers, which amplify bacterial 16S rDNA sequences while simultaneously avoiding the amplification of organellar DNA sequences. Thus, several

¹<https://ec.europa.eu/eurostat>

primer pairs have been developed with that purpose, revealing different performances depending of the study (e.g., Chelius and Triplett, 2001; Sogin et al., 2006; Walker and Pace, 2007; Beckers et al., 2016; Dos Santos et al., 2017). However, the experimental performance of these mismatch primers and their efficacy in reducing co-amplification of non-target DNA in different plant species or plant compartments that may differ on organellar input have not been evaluated enough. Consequently, it is essential to evaluate the amplification efficiency and robustness of selected primer pairs in plant–bacteria interaction studies to assess their behavior in different host plants and specific plant compartments, especially on those rarely addressed such as the xylem tissue.

To our knowledge, no study has systematically evaluated the effects of different DNA extraction methods and the choice of primer pairs to conduct studies on xylem sap bacterial microbiota, which is a key information that is still missing. Consequently, the objectives of this work have been: (1) to compare several standard DNA extraction kits and primer pairs targeting different hypervariable regions of the 16S rRNA gene and described as suitable to avoid co-amplification of plant organellar rRNA gene sequences for their efficacy in the description of the structure and diversity of xylem sap bacterial microbiota when coupled with NGS and bioinformatic tools; (2) to assess whether one of the selected DNA extraction methods combined with different primer pairs could provide an accurate representation of bacterial communities using a mock microbial community standard; and (3) to demonstrate the utility of the selected protocol for assessing differences in xylem bacterial composition of two of the most widely grown olive cultivars in Spain.

MATERIALS AND METHODS

Xylem Sap Collection

Xylem sap extraction from olive branches was performed using a Scholander pressure chamber pressurized with compressed nitrogen and coupled with an external 60-cm-long super chamber following the Bollard process and as described by Anguita-Maeso et al. (2020). Shortly, xylem sap was extracted from 30-cm-long, 2-year-old branches of adult olive trees, which were approximately 1 cm in diameter in their thicker part and which were debarked at the external part to avoid contamination by phloem fluids. Pressure was increased progressively up to a maximum of 35 bar (3.5 MPa), with the first few drops of sap being discarded; then the xylem sap was collected into 5-ml Eppendorf tubes kept on ice for 20–30 min until a minimum volume of 4.0 ml per branch was obtained. Xylem sap was kept at -80°C prior to DNA extraction. All the processes described above took place under sterile conditions into a flow hood chamber.

Extraction of Microbial DNA From Xylem Sap

For the initial experiment to evaluate different microbial DNA extraction protocols, a total of 15 olive branches were used

from four 8-year-old olive trees of cv. “Picual.” The xylem sap extracted from all branches was combined into a composite sample, vortexed to homogenize it, and then split into 3-ml aliquots. Samples were then centrifuged at $12,000 \times g$ for 20 min at 4°C . The supernatant was removed into a new tube, and the pellet and the supernatant were stored at -20°C separately.

Xylem sap pellets were used for total DNA extraction using 11 commercial microbial DNA extraction kits following the protocol as indicated by the manufacturer or with slight modifications (Table 1). Additionally, the cetyltrimethylammonium bromide (CTAB) protocol (2% hexadecyltrimethylammonium bromide, 0.1 M Tris–HCl pH 8, 20 mM EDTA, and 1.4 M NaCl) was included. Two replicates of xylem sap samples were used for each protocol. Before starting each protocol, the pellet was resuspended in the corresponding extraction buffer of each protocol by vortexing briefly. DNA was eluted in a final volume of 50 μl of ultrapure, filtered-sterilized distilled water. For each protocol and replicated sample, the following parameters were determined: (1) the yield and purity of the DNA (absorbance 260/280 nm ratio) by using a NanoDrop[®]156 ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, United States), (2) the PCR yield using PCR primers 967F/1193R and agarose gel electrophoresis (see below), (3) time of processing, and (4) cost (Table 1).

Validation of a DNA Extraction Protocol and PCR Primers

Microbial composition profiling techniques powered by NGS can suffer from significant bias at different steps from bacterial sampling to bioinformatic analysis workflows. A mock standard microbial community was used to validate which of the four primer pair combinations could provide the most accurate representation of the microbial communities present in the xylem sap and to which extent the selection of the reference database for taxonomic assignment could also influence results. The ZymoBIOMICS microbial standard (Zymo Research) was used as a mock standard microbial community. This is a well-defined, accurately characterized mock community consisting of three easy-to-lyse bacteria, five tough-to-lyse bacteria, and two tough-to-lyse yeasts. Its theoretical bacterial composition according to the manufacturer based on 16S rRNA gene abundance is *Listeria monocytogenes*, 14.1%; *Pseudomonas aeruginosa*, 4.2%; *Bacillus subtilis*, 17.4%; *Escherichia coli*, 10.1%; *Salmonella enterica*, 10.4%; *Lactobacillus fermentum*, 18.4%; *Enterococcus faecalis*, 9.9%; and *Staphylococcus aureus*, 15.5%.

The PowerPlant DNA extraction method was selected to verify its efficiency at extracting accurate representative quantities of DNA from both Gram-positive and Gram-negative bacteria present in the mock microbial community standard (MC). Mock sap samples were prepared using 3 ml of bacterial-free xylem sap (previously filtered through a 0.22- μm filter and showing no amplification of 16S rRNA gene with PCR1, see below) to which we added 50 μl of ZymoBIOMICS Microbial Community Standard Cells (Zymo Research Corp., Irvine, CA, United States). Mock sap samples were then processed as indicated above for the

TABLE 1 | Characteristics of the DNA extraction protocols used in the study.

ID Protocol	Protocol ^a	Trademark	DNA yield (ng/ μ l)	Absorbance 260/280	Manufacturer's instructions procedure	Amplification ^b 16S	Price to 50 preps ^c (€)	Extraction time (min)
PowerPlant	DNeasy PowerPlant Pro Kit	Qiagen	5.9 \pm 1.4	1.7	Yes	+++	4.0	40
PowerSoil	DNeasy PowerLyzer PowerSoil kit	Qiagen	2.7 \pm 0.1	1.7	Yes	++	7.3	50
MoBioSoil	PowerSoil [®] DNA Isolation Kit	Mo Bio	5.4 \pm 2.8	1.3	Yes	+	5.3	55
PureLink	PureLink [™] Microbiome DNA Purification Kit	Invitrogen	8.5 \pm 3.4	1.4	Yes	+	5.4	50
NorgenMicrobiomeV1	Microbiome DNA Isolation kit	Norgen	1.6 \pm 0.3	1.3	Yes	++	4.0	65
NorgenMicrobiomeV2	Microbiome DNA Isolation kit	Norgen	16.7 \pm 1.5	2.0	Yes, using Binding Buffer B instead of Binding Buffer I	+++	4.0	65
QuickPick	QuickPick [™] SML Plant DNA	Bio-Nobile	16.6 \pm 0.1	2.5	Yes	+	2.3	70
CTAB	CTAB ^c		1.0 \pm 0.5	1.8	Yes	++	1.0	105
NucleoSpinPL1	NucleoSpin [®] Plant II	Macherey-Nagel	3.1 \pm 1.4	1.9	Yes, using PL1 lysis buffer	+	3.2	80
NucleoSpinPL2	NucleoSpin [®] Plant II	Macherey-Nagel	1.1 \pm 0.7	1.1	Yes, using PL2 lysis buffer	+	3.2	95
CanvaxSoil	HigherPurity [™] Soil DNA Isolation Kit	Canvax Biotech	5.9 \pm 3.7	1.4	Yes	+++	5.6	70
CanvaxTissue	HigherPurity [™] Tissue DNA Purification Kit	Canvax Biotech	2.6 \pm 0.4	2.3	Yes	++	2.4	95

^aCommercial kit name. CTAB, cetyltrimethylammonium bromide.

^bRelative amplification as measured by the intensity of the amplified product after agarose gel electrophoresis visualization: (+++) = very good, (++) = good, (+) = weak.

^cTimes that the cost for each kit is more expensive than the CTAB cost for extracting 50 samples.

^dSample preparation time not including sap extraction.

PowerPlant DNA extraction protocol. After DNA extraction, the four PCR protocols described below were evaluated. There were four replicates per combination of PCR protocol.

Sampling Xylem From Olive Cultivars and Extracting DNA

Olive orchards from an experimental plot located at the Institute for Sustainable Agriculture from Spanish National Research Council (IAS-CSIC) facilities in Córdoba (southern Spain) were used to test the differences in xylem bacterial composition between two of the most widely grown olive cultivars in Spain. The orchard was established in September of 2014, with 2-year-old olive trees of cultivar “Picual” and “Arbequina” propagated at “Plantas Continental S.A.” nursery (Ribero de Posadas, Córdoba, Spain). Both genotypes were planted in the orchard with a random block design and received similar growing practices until sampling.

Seven trees of each cultivar were sampled in May 2017, and xylem sap was extracted as described above. The average sap volume extracted ranged from 3.5 to 4 ml per sampled branch. In this case and to facilitate DNA extraction, the sap samples were immediately filtered through a 0.22- μ m Millipore filter, and the filters retaining all microbial cells contained in the sap and the filtered sap were stored independently at -20°C or -80°C , respectively. DNA extraction was performed using the PowerPlant protocol. First, the filtered cells were

resuspended in the extraction buffer by vortexing briefly, and then the microbial suspension was processed following the manufacturer's instructions (Table 1). To analyze bacterial communities, DNA samples were amplified using the four PCR protocols described below.

16S rRNA Gene Amplification

Four PCR protocols using five primer pairs targeting different hypervariable regions of the 16S rRNA gene were compared to evaluate their performance in metabarcoding studies of xylem sap bacterial communities. Those primer pairs have been described in previous studies as appropriate to avoid co-amplification of plant chloroplast and mitochondrial DNA. For that, two primer pairs were used in two direct PCR protocols, and additionally, three primer pairs were used in two nested PCR protocols to evaluate if an increase in amplification yield could be obtained by the nested PCR approach (Table 2):

PCR1-(799F/1391R) uses primers 799F and 1391R that amplify a 600-bp fragment of the V5–V8 region of the bacterial 16S rRNA gene (Chelius and Triplett, 2001; Walker and Pace, 2007; Beckers et al., 2016), and its amplification product is used as a template for the second round of nested PCRs described below.

PCR2-(967F/1193R) uses primers 967F and 1193R that amplify the V6–V7 region of the bacterial 16S gene (Dos Santos et al., 2017). This PCR was initially selected to test the different

TABLE 2 | PCR protocols used in the study, with primer sequences, hypervariable region of 16S rRNA gene amplified, and expected product size.

PCR	Primer pairs	Primer forward	Primer reverse	Region	Size (pb)	References
PCR-1	799F + 1391R	AACMGATTAGATACCKG	GACGGGCGGTGWGTRCA	V5–V8	600	Walker and Pace, 2007; Beckers et al., 2016; Dos Santos et al., 2017
PCR-2	967F + 1193R	CAACGCGAAGAACCTTACC	ACGTCATCCCCACCTTCC	V6–V7	230	Bodenhausen et al., 2013; Dos Santos et al., 2017
PCR-3	799F + 1193R	AACMGATTAGATACCKG	ACGTCATCCCCACCTTCC	V5–V7	400	Sogin et al., 2006; Walker and Pace, 2007; Beckers et al., 2016
N1PCR1*	799F + 1193R	AACMGATTAGATACCKG	ACGTCATCCCCACCTTCC	V5–V7	400	Sogin et al., 2006; Walker and Pace, 2007; Beckers et al., 2016
N2PCR1*	967F + 1391R	CAACGCGAAGAACCTTACC	GACGGGCGGTGWGTRCA	V6–V8	430	Walker and Pace, 2007; Callahan et al., 2016; Dos Santos et al., 2017

*N1PCR1 and N2PCR1 were nested PCRs that used as DNA template the amplification product obtained with primers 799F + 1193R (PCR1).

DNA extraction protocols, and it was also included to compare its performance with the other three PCR protocols described below.

PCR3-(799F/1193R) uses primers 799F and 1193R that amplify the V5–V7 region of the bacterial 16S rRNA gene (Chelius and Triplett, 2001; Bodenhausen et al., 2013; Beckers et al., 2016).

N1PCR1-(799F/1391R + 799F/1193R) uses primers 799F and 1391R in the first round of PCR (i.e., PCR1) and primers 799F and 1193R in the second round of PCR.

N2PCR1-(799F/1391R + 967F/1391R) uses primers 799F and 1391R in the first round of PCR (i.e., PCR1) and primers 967F and 1391R that amplify the V6–V8 region of the bacterial 16S rRNA gene in the second round of PCR (Sogin et al., 2006; Walker and Pace, 2007; Beckers et al., 2016).

All PCRs were carried out in 25- μ l reaction volumes containing 0.05 units of MyTaqTM DNA Polymerase (Bioline Laboratories, London, United Kingdom), 1 \times MyTaqTM Mix (Bioline) and forward and reverse primers at a concentration of 0.3 μ M each, and 3 μ l of template DNA. PCR protocol consisted of an initial denaturalization step at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 53°C for 45 s, 72°C for 1 min, and a final elongation step at 72°C for 8 min. For the nested PCR approach, 1 μ l of the amplification product of the first reaction was used as the template for the second PCR that was performed with the same conditions as those of the first PCR.

Library Preparation and Sequencing

PCR amplicons were cleaned up before adaptor addition using the Ampure XP magnetic bead system (Beckmann Coulter, MA, United States) according to manufacturer's recommendation. Dual barcode indices and sequencing adaptors were attached to each amplicon using the Illumina Nextera XT Index kit (Illumina, Inc., San Diego, CA, United States) following the manufacturer's protocol, followed by a further Ampure XP cleanup step. Purified amplicons were quantified using the Quant-iTTM PicoGreenTM dsDNA Assay Kit (Thermo Fisher Scientific) and a Tecan Safire microplate reader (Tecan Group, Männedorf, Switzerland). Equimolecular amounts from each individual sample in 10 mM of Tris were combined, and the pooled library was additionally purified with two rounds of Ampure XP cleanup step. The library was sequenced by the Genomics Unit at "Fundación Parque Científico de Madrid"

(Madrid, Spain) using the Illumina MiSeq platform (Nano-V2; PE 2x 250 bp). The ZymoBIOMICS microbial standard (Zymo Research Corp., Irvine, CA, United States) and water (no template DNA) were used as internal positive and negative controls, respectively, for library construction and sequencing. Raw sequence data have been deposited in the Sequence Read Archive (SRA) database at the NCBI under BioProject accession number PRJNA684121.

Data Processing and Bioinformatic Analysis

16S rRNA gene sequences were analyzed and classified using the Quantitative Insights into Microbial Ecology bioinformatic pipeline, QIIME2 (version 2019.10²; Caporaso et al., 2010; Bolyen et al., 2018) with default parameters unless otherwise noted. Demultiplexed sequences were imported as CASAVA format. Sequence quality control, denoising, and chimeric filtering were performed with DADA2 pipeline (Callahan et al., 2016). Taxonomy affiliation was identified by operational taxonomic units (OTUs) at 99% similarity using VSEARCH consensus taxonomy classifier (Rognes et al., 2016) based on Greengenes_13_8_99 (DeSantis et al., 2006; McDonald et al., 2011) and Silva_132_99 (Quast et al., 2013; Yilmaz et al., 2014) reference databases. Singletons were discarded for downstream analysis.

Alpha diversity and beta diversity as well as alpha rarefaction curves were conducted rarefying all samples to the minimum number of reads found. Sequencing depth was of 2,276 and 2,489 for the evaluation of the DNA extraction protocols and the microbiota of the olive tree cultivars, respectively. Rarefaction curves and alpha diversity indexes (Shannon and Richness or number of observed OTUs) were performed using the OTU frequency matrixes at the OTU level with the online tool MicrobiomeAnalyst (Chong et al., 2020)³. The Kruskal–Wallis test ($P < 0.05$) with false discovery rate (FDR) correction (Benjamini and Hochberg, 1995) was used to determine the effects of the factors in the study on alpha diversity. To analyze the association of PCR protocols and olive genotypes with these alpha diversity matrices, we applied general linear modeling (GLM)

²<https://view.qiime2.org/>

³<https://www.microbiomeanalyst.ca>

using the lme4 package in R (Bates et al., 2015), with a two-factor factorial design, being the PCR protocols [967F-1193R (PCR2), $n = 14$; 799F-1193R (PCR3), $n = 14$; 799F-1391R + 799F-1193R (N1PCR1), $n = 14$; 799F-1391R + 967F-1391R (N2PCR1), $n = 14$] and the olive genotype (“Arbequina”; $n = 28$; “Picual,” $n = 28$) the two main factors. Venn diagrams were generated using the “Venn diagram” online tool⁴ and were used to identify shared (core microbiota) or unique taxa according to the DNA extraction protocol or the PCR primers used.

For beta diversity analysis, we performed multivariate hierarchical clustering analysis (using Bray–Curtis index to measure distance and the Ward clustering algorithm), as well as non-supervised principal coordinate analysis (PCoA) using Bray–Curtis distance (a non-phylogenetic metric) (Beals, 1984), and the weighted UniFrac distances (a phylogenetic metric) (Lozupone and Knight, 2005) were performed using the OTU frequency matrixes at the OTU level with QIIME2 to test for similarities among the bacterial communities according to the DNA extraction protocol or among the PCR protocols and between the olive genotypes. In addition, the PERMANOVA test ($P < 0.05$) was used to determine the effects of those factors.

The theoretical relative abundance of the ZymoBIOMICS Microbial Community Standard was compared with the estimated relative abundances of the identified OTUs at the genus level that were obtained for each PCR and reference database (Silva or Greengenes) using Spearman’s correlation analysis (McGovern et al., 2018). Similarity was considered as significant if P value < 0.05 and as a trend if $0.05 \geq P$ value ≤ 0.1 .

Finally, to analyze in further detail the differences in microbiota composition at the genus level among the different PCR protocols, a negative binomial model approach based on the DESeq2 package in R (Love et al., 2014) was used. Wald tests were performed, and only genera remaining significant ($P < 0.01$) were retained.

RESULTS

Effect of DNA Extraction Protocols on Xylem Sap Bacterial Community Assessment

In the bacterial community analyses of the 12 different DNA extraction protocols tested, a total of 108,236 high-quality 16S rRNA gene paired-end sequences with an average of 4,510 sequences per DNA protocol were retained after discarding poor-quality sequences. From those, approximately 56% could be classified into bacterial OTUs with a mean of 2,460 and 2,468 bacterial sequences in Greengenes_13.8 or Silva_132 databases, respectively (Table 3). However, the number of plant organellar rRNA gene sequences amplified varied according to the DNA extraction kit, with the NorgenMicrobiomeV2 and PureLink DNA extraction kits showing the highest proportion of organellar reads ($\geq 70\%$) and both NucleoSpin and the PowerLyzer DNA extraction kits showing the lowest proportion ($\leq 4\%$), independently of the reference database used for taxonomic

assignment (Table 3). A total of 209 or 248 OTUs, with an average number of 58 or 60 OTUs, were identified as bacteria when using the Greengenes_13.8 or Silva_132 databases, respectively (Table 3), with the higher number of OTUs being identified when using the NucleoSpin and PowerLyzer DNA extraction kits (i.e., identified OTUs ranged between 84 and 93) (Table 3).

Rarefaction curves of observed OTUs (Richness) indicated a good sequencing coverage among all DNA extraction protocols, with no differences between both reference databases (Supplementary Figure 1). DNA extraction protocol significantly affected xylem sap bacterial community assessment. Thus, Richness alpha diversity index showed significant differences ($P < 0.044$) among the different DNA extraction methods, whereas no significant differences ($P > 0.091$) were found for the Shannon index regardless of the taxonomy database used (Figure 1). On the other hand, hierarchical clustering analysis and PCoA of Bray–Curtis index and weighted UniFrac distances using OTU frequencies differentiated xylem bacterial communities in four clusters according to the DNA extraction method regardless of the database used for taxonomy assignment (Figure 2A). Thus, most DNA extraction kits from four different brands clustered together (Group 4), whereas the CTAB protocol (Group 1), both Canvax kits (Group 2), and the two NorgenMicrobiome protocols (Group 3) clustered independently from each other, although Group 1 and Group 2 clustered closer and apart from the other DNA extraction kits (Figure 2A). Similarly, PCoA of Bray–Curtis and weighted UniFrac distances differentiated xylem bacterial communities according to the DNA extraction kits in the four groups (Figure 2B) regardless of the database used. PERMANOVA indicated a significant clustering due to the DNA extraction protocol used ($pseudo-F < 12.386$; $P = 0.001$). This clustering, derived from beta diversity analyses, of the 12 DNA extraction kits in four independent groups was used to summarize the influence of DNA extraction kits on assessing the composition of bacterial communities in olive xylem sap.

A total of 13 or 16 phyla, 29 or 34 classes, 53 or 65 orders, 118 or 124 families, and 209 or 248 genera were identified when using the Greengenes or Silva database, respectively (Supplementary Figure 2). When comparing results from both databases, the core number of taxa detected by all the DNA extraction kits was four phyla, seven classes, and 12 orders, whereas 16 or 21 families and 12 or 14 genera were detected when using the Greengenes or Silva database, respectively. The lowest numbers of phyla and classes were identified when using the Canvax DNA extraction kits (Group 2), whereas the lowest numbers of orders, families, and genera were identified with the CTAB DNA extraction protocol (Group 1). In all cases, the highest numbers of total and unique phyla, orders, classes, families, and genera were identified for the DNA kits included in Group 4. Finally, the DNA extraction kits included in Groups 3 and 4 shared the highest number of bacterial taxa (Supplementary Figure 2).

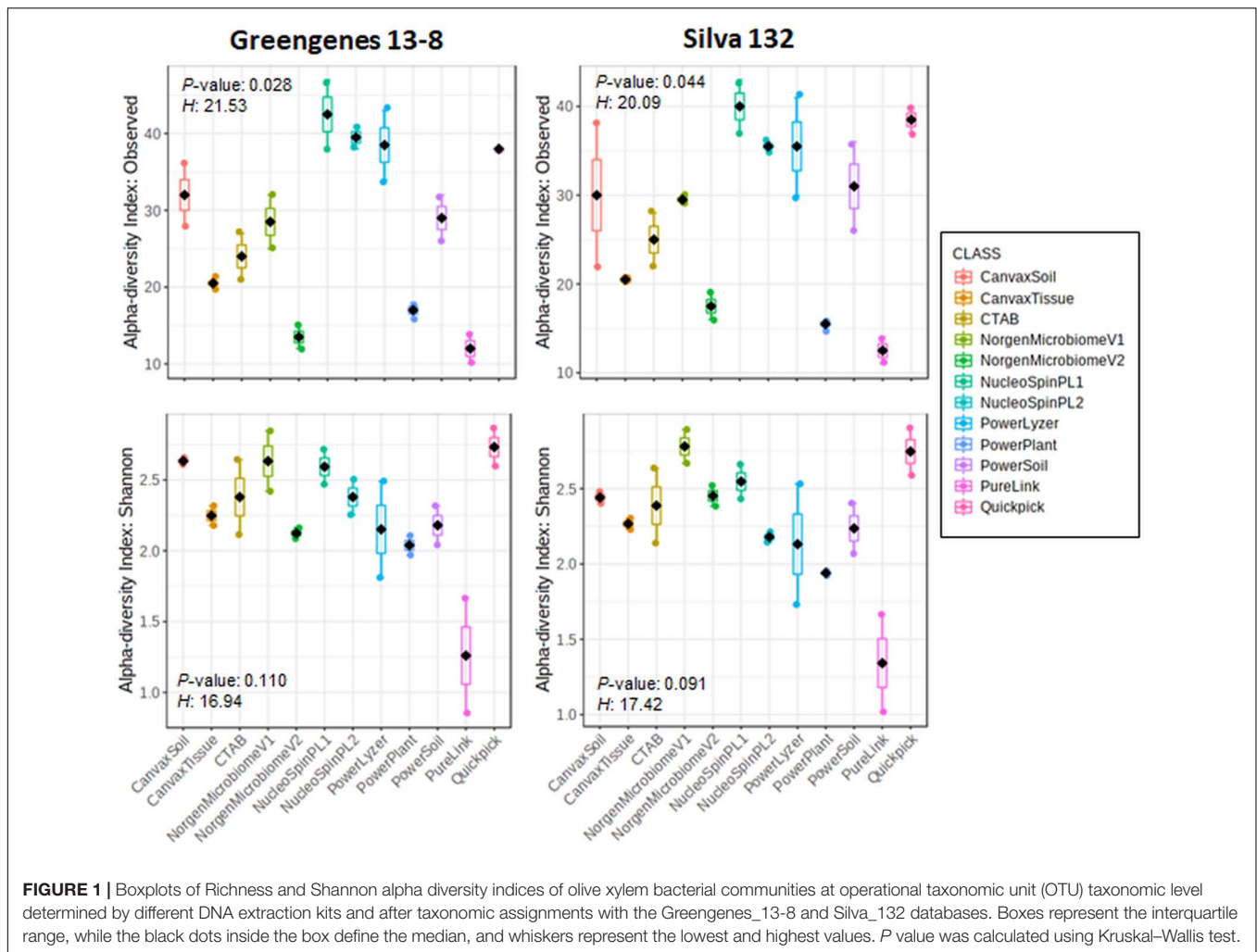
Four phyla including Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes were the most abundant and were identified by all DNA extraction kits, representing 51.8%, 26.2%, 9.7%, and 9.6% of the total, respectively (Figure 3 and Supplementary Figure 2). In addition,

⁴<http://bioinformatics.psb.ugent.be/webtools/Venn/>

TABLE 3 | Number of reads and operational taxonomic units (OTUs) derived from NGS analysis of xylem sap microbiota from DNA samples extracted with 12 extraction protocols, amplified with the 967F + 1193R primer pair (PCR2), and taxonomic assignment using the Greengenes 13-8 and Silva 132 reference databases.

Reads/OTU numbers		Norgen MicrobiomeV1	Norgen MicrobiomeV2	Pure Link	Quick Pick	Power Soil	Power Lyzer	Power Plant	Nucleo Nucleo	Nucleo SpinPL2	Canvax Soil	Canvax Tissue	CTAB*
Number of reads (Mean/sample)		4,586	5,888	3,931	4,977	4,724	4,502	3,265	3,828	4,731	5,777	4,090	3,821
Greengenes	Unassigned Reads (%)	15	11	8	12	13	8	11	46	21	5	13	6
	Plant (chloroplast + mitochondria) reads (%)	33	70	79	18	18	3	28	2	4	59	36	14
	Bacterial reads (%)	52	19	13	70	69	89	61	52	75	36	51	80
	OTUs at the Phylum level	7	6	4	7	7	8	7	8	7	6	5	8
	OTUs at the Class level	14	7	6	12	9	14	13	12	14	10	7	12
	OTUs at the Order level	25	10	12	24	16	24	21	26	28	18	12	19
	OTUs at the Family level	42	17	19	48	34	49	41	56	58	42	30	35
	OTUs at the Genus level	51	24	21	66	41	75	54	81	77	55	32	45
Total Assigned OTUs	56	24	21	73	50	90	63	91	88	62	35	47	
Silva	Unassigned Reads (%)	15	11	8	11	13	7	11	46	21	5	13	6
	Plant (chloroplast + mitochondria) reads (%)	33	70	79	18	18	3	28	2	4	59	36	14
	Bacterial reads (%)	52	19	13	71	69	90	61	52	75	36	51	80
	OTUs at the Phylum level	8	6	4	8	8	10	8	9	9	6	6	9
	OTUs at the Class level	16	7	6	14	11	17	15	14	19	10	9	14
	OTUs at the Order level	31	15	14	30	20	30	28	32	37	21	16	23
	OTUs at the Family level	47	21	19	49	35	52	42	52	60	39	30	39
	OTUs at the Genus level	60	28	21	67	45	80	58	78	82	54	33	49
Total Assigned OTUs	64	29	21	74	49	93	65	84	91	61	35	52	

CTAB, cetyltrimethylammonium bromide.

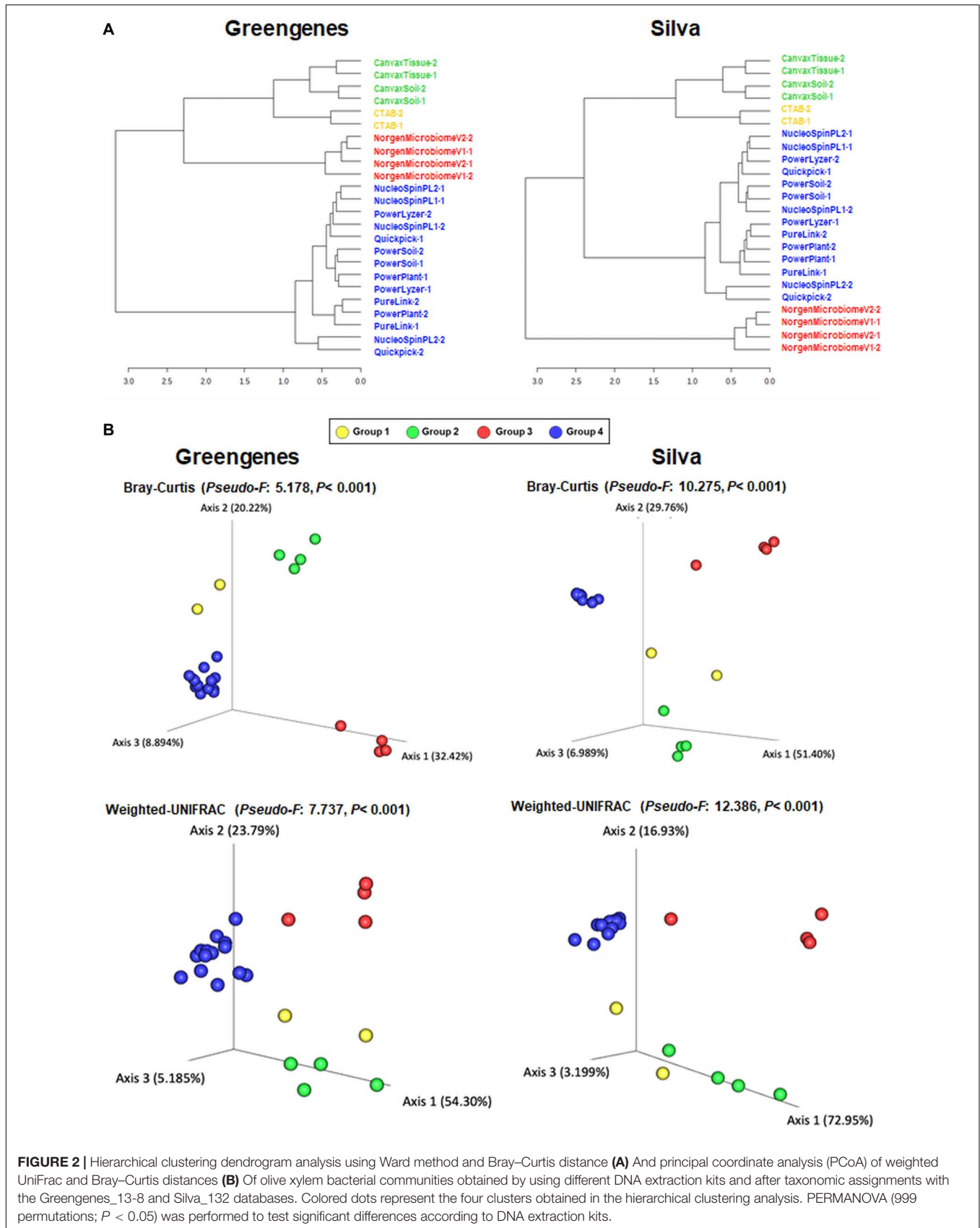


three minority phyla (<0.11% abundance), Nitrospirae, Fibrobacteres, and Chloroflexi, were only identified when using the NorgenMicrobiome DNA extraction kits (Group 3), whereas three other phyla, Saccharibacteria, Gemmatimonadetes, and Verrucomicrobia, showed a low frequency (0.2%) and were only identified when using the different DNA extraction kits from Group 4 (**Supplementary Figure 2**). Seven bacterial classes were detected by all DNA extraction kits, with Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Bacilli, and Gammaproteobacteria being the most abundant, representing 26.1%, 22.6%, 19.7%, 9.0%, and 8.8% of the total, respectively (**Figure 3** and **Supplementary Figure 2**). At the family level, 16 or 21 families, depending on the reference database used, comprised the core bacterial microbiota of all DNA extraction kits, being Propionibacteriaceae, Bradyrhizobiaceae, Comamonadaceae, and Chitinophagaceae the most abundant (mean frequency ranged between 5.9 and 18%) (**Figure 3** and **Supplementary Figure 2**). Finally, at the genus level, 12 or 14 genera, depending of the reference database (**Supplementary Figure 2**), comprised the core bacterial microbiota, being *Propionibacterium*, and unidentified Comamonadaceae, *Sediminibacterium*, and

unidentified Rhizobiales, and unidentified Methylophilaceae, and unidentified Bradyrhizobiaceae, *Novosphingobium*, *Staphylococcus*, *Bradyrhizobium*, and *Flavobacterium* the most abundant genera (mean frequencies ranged from 18.8 to 3.0%) by all DNA extraction kits when using the Greengenes database; whereas with the Silva database, *Propionibacterium*, *Bradyrhizobium*, an unidentified Comamonadaceae, an unidentified Chitinophagaceae, an unidentified Methylophilaceae, *Novosphingobium*, *Bosea*, *Flavobacterium*, *Staphylococcus*, and *Pseudomonas* were the most abundant genera (mean frequencies ranged from 18.7 to 2.8%). Interestingly, up to 114 genera could be detected uniquely by the DNA extraction kits within Group 4.

Validation of a DNA Extraction Method, Primer Pair Performance, and Taxonomy Reference Databases

In this study, we used a mock microbial community standard to determine if a selected DNA extraction kit (the PowerPlant from Qiagen) within Group 4 (the one providing the highest values of alpha diversity) provides an accurate representation of



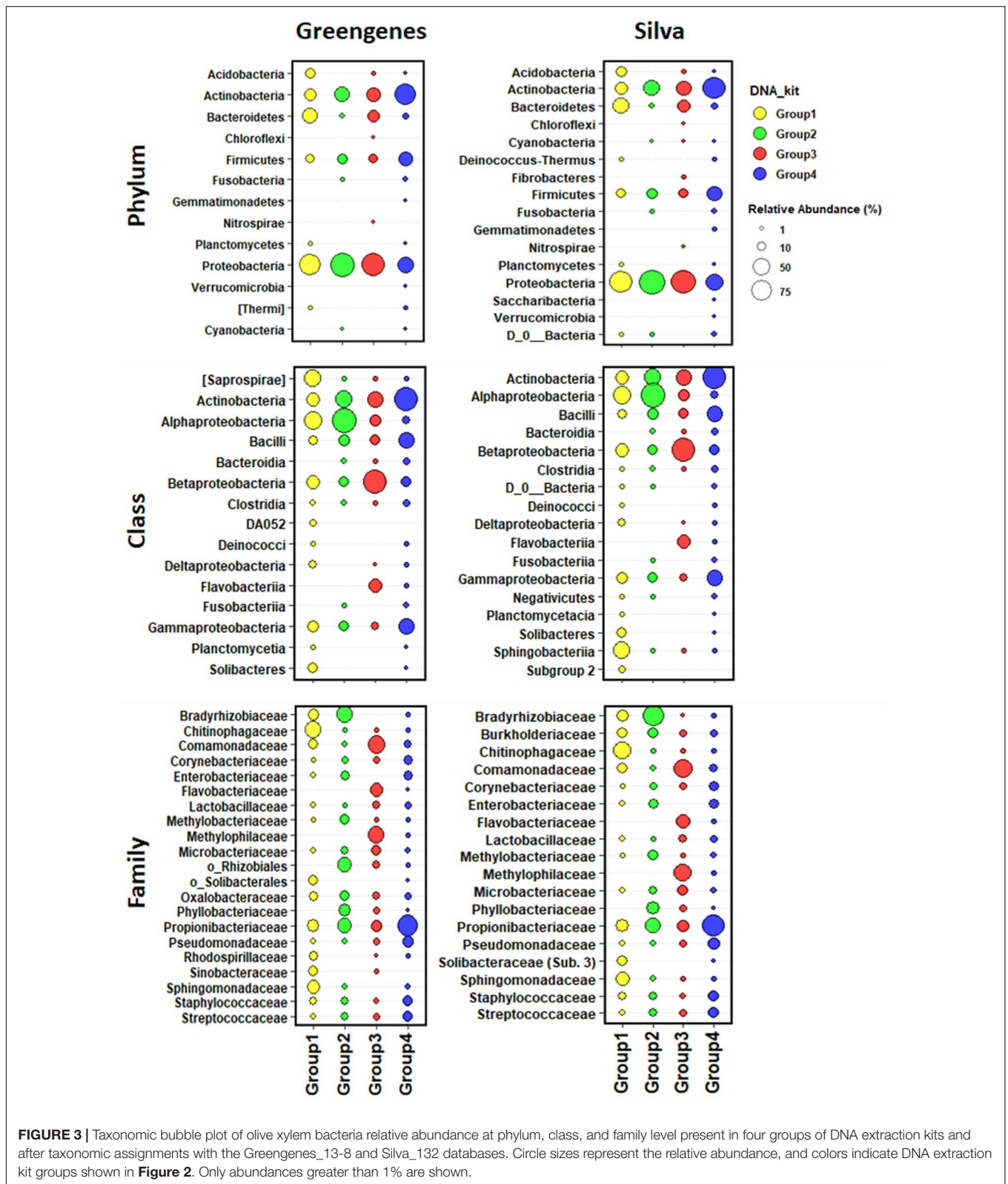


FIGURE 3 | Taxonomic bubble plot of olive xylem bacteria relative abundance at phylum, class, and family level present in four groups of DNA extraction kits and after taxonomic assignments with the Greengenes_13-8 and Silva_132 databases. Circle sizes represent the relative abundance, and colors indicate DNA extraction kit groups shown in **Figure 2**. Only abundances greater than 1% are shown.

the identified microbial communities and if this can be affected by the PCR protocol and the reference database used for taxonomic assignment (Greengenes 13-8 and Silva 132).

We found a very small background amplification of other OTUs or genera other than the eight expected to be present in the ZYMO mock community. Background amplification was

observed on all samples and PCRs when Silva database was used and represented about 9.8–32.6% of reads. Among the background bacteria amplified, unidentified OTUs belonging to the Class Bacilli, the Order Lactobacillales, and the genus *Granulicatella* were detected, although most of the “contaminant” reads were assigned only as Bacteria and could not be assigned taxonomically to any phylum. On the other hand, when using Greengenes database, background amplification was detected only on PCR2 and N1PCR1 but represented less than 2% of reads, with only the genus *Granulicatella* being detected.

All eight expected bacterial genera were detected by the four PCR protocols used regardless of the reference database used (Figure 4). However, there was a large effect on accuracy of genus relative abundance estimation depending on the PCR and the reference database used. Thus, only when using primer pair 799F/1193R (PCR3) and when performing the taxonomic assignment with the Silva 132 database was a significant correlation (Spearman coefficient 0.714, $P = 0.046$) found between the theoretical and the estimated bacterial community composition (Figure 4). Consequently, we decided to select the Silva database for taxonomic assignment for further analysis.

Effect of Primer Pairs on Xylem Sap Bacterial Community Assessment of Two Olive Cultivars

For the bacterial community analyses of the 14 samples from “Picual” and “Arbequina” olive trees, after screening our data for

poor-quality sequences and removing chimeras and unassigned reads, we recovered a total of 86,489, 54,143, 44,444, and 33,261 high-quality paired-end sequences with the PCR2, PCR3, N1PCR1, and N2PCR1 protocols, respectively; with an average of 6,178, 3,867, 3,175, and 2,376 sequences per PCR, respectively (Table 4). The number of unassigned reads was lower (<3%) for the two direct PCRs (PCR2 and PCR3) as compared to the two nested PCRs (>6.5%). However, and only for primers 967F-1193R (PCR2), a high percentage of reads were of plant origin, whereas no such amplification occurred with the other PCR protocols (Table 4).

For Richness alpha diversity index, we found a significant effect for the PCR protocol ($P < 0.001$) and no effect for olive genotype ($P = 0.537$). For Shannon diversity index, no significant effect was found for any of the factors ($P > 0.554$) (Figure 5). In general, both nested PCR approaches showed lower values of alpha diversity indexes (Figure 5). To determine whether the choice of PCR approach (primer pair) influenced microbial community composition, we calculated two beta diversity metrics (Bray–Curtis and weighted UniFrac) and included the PCR protocol, olive genotype, and the interaction PCR protocol \times olive genotype as explanatory variables in PERMANOVA models. In these analyses, microbial beta diversity estimates were significantly influenced by the PCR protocol (Bray–Curtis: pseudo- $F = 22.12$, $P < 0.001$; weighted-UniFrac: pseudo- $F = 11.96$, $P < 0.001$), as well by the interaction PCR protocol \times genotype (Bray–Curtis: pseudo- $F = 10.22$, $P < 0.001$; weighted-UniFrac: pseudo- $F = 5.93$, $P < 0.001$), but not by

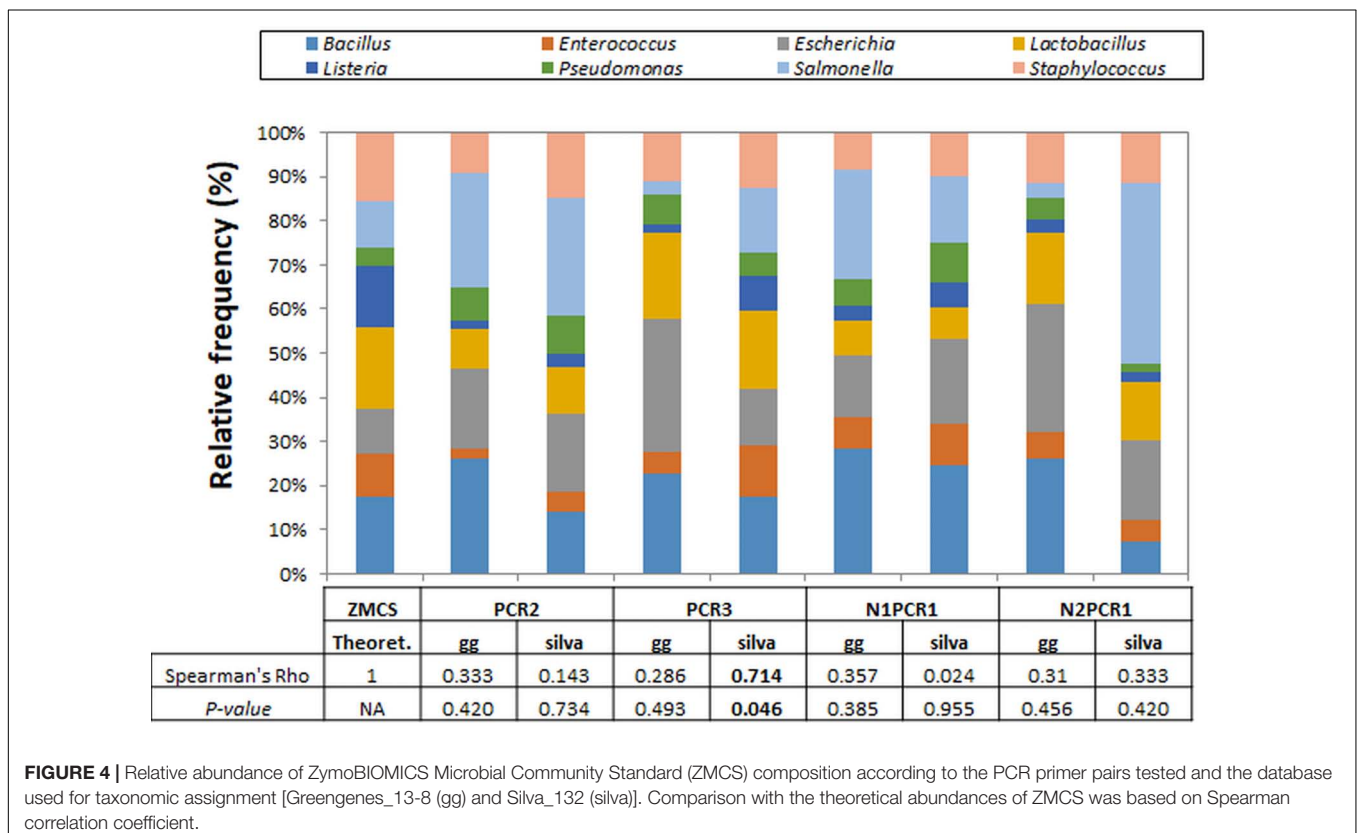


FIGURE 4 | Relative abundance of ZymoBIOMICS Microbial Community Standard (ZMCS) composition according to the PCR primer pairs tested and the database used for taxonomic assignment [Greengenes_13-8 (gg) and Silva_132 (silva)]. Comparison with the theoretical abundances of ZMCS was based on Spearman correlation coefficient.

TABLE 4 | Number of reads and OTUs derived from NGS analysis of xylem sap microbiota from DNA samples extracted with the PowerPlant protocol, amplified with four PCR protocols, and taxonomic assignment using the Silva 132 reference database.

Reads/OTUs	PCR-2	PCR-3	N1PCR1*	N2PCR1*
	967F-1193R	799F-1193R	799F-1193R	967F-1391R
Number of reads (Mean/sample)	6178	3867	3175	2376
Unassigned Reads (%)	2.06	2.89	12.09	6.58
Plant (chloroplast + mitochondria) reads (%)	56.75	0	0	0
Bacterial reads (%)	41.19	97.11	87.91	93.42
OTUs at the Phylum level	13	10	8	7
OTUs at the Class level	27	17	13	13
OTUs at the Order level	52	47	39	39
OTUs at the Family level	94	93	68	70
OTUs at the Genus level	154	162	100	107
Total Assigned OTUs	233	242	141	156

*N1PCR1 and N2PCR1 were nested PCRs that used as DNA template the amplification product obtained with primers 799F + 1193R (PCR1) (Table 2). NGS, next-generation sequencing; OTU, operational taxonomic unit.

the olive genotype (Bray–Curtis: pseudo- $F = 0.53$, $P = 0.90$; weighted-UniFrac: pseudo- $F = 1.61$, $P = 0.118$) on microbial beta diversity estimates (Figure 6). N1PCR1, which is a nested

PCR that uses in the second round of PCR the same primers as those used in PCR3, clustered and grouped together and closer to samples amplified with this later PCR both in the hierarchical cluster analysis (Figure 6A) and in the PCoA using Bray–Curtis distance (Figure 6B). According to the phylogenetic distances among the bacterial communities amplified by each PCR protocol, primers 967F-1193R (PCR2) showed a more distinct bacterial community composition as compared to PCR3, N1PCR1, and N2PCR1 that tended to overlap (Figure 6B).

The differences on alpha and beta diversity indexes found among PCR protocols were due to differences on both the number of bacterial taxa identified and their abundances. In fact, a total of 8–13 phyla, 13–27 classes, 39–52 orders, 68–94 families, 100–162 genera, and 141–242 species were identified depending of the PCR protocol (Table 4 and Supplementary Figure 3). The core number of taxa detected by all the PCR protocols included six phyla, nine classes, 28 orders, 48 families, and 50 genera. The lowest numbers of all bacterial taxa were obtained for both nested PCR protocols, whereas the highest numbers of phyla, classes, and orders were detected with primers 967F-1193R (PCR2), and those of genera and species were obtained with primers 799F-1193R (PCR3). In all cases, the highest numbers of unique and shared taxa occurred for both direct PCR protocols (PCR2 and PCR3) (Table 4 and Supplementary Figure 3).

In general, the bacterial taxa with higher abundance were detected by all the four PCR protocols (Figure 7 and Supplementary Figure 3). At phylum level, Actinobacteria, Proteobacteria, Firmicutes, and Deinococcus-Thermus represented 51.9%, 29.0%, 16.8%, and 1.2%, respectively. Six bacterial classes were detected by all PCR protocols, with Actinobacteria, Gammaproteobacteria, Bacilli, Alphaproteobacteria, Clostridia, and Deinococci being the most abundant, representing 51.6%, 23.4%, 15.6%, 5.5%, 1.2%, and 1.2%, respectively (Figure 7 and Supplementary Figure 3). Fourteen families were detected by all PCR protocols, with Propionibacteriaceae, Staphylococcaceae, Pseudomonadaceae, Burkholderiaceae, Enterobacteriaceae, Sphingomonadaceae, Corynebacteriaceae, Microbacteriaceae being the most abundant

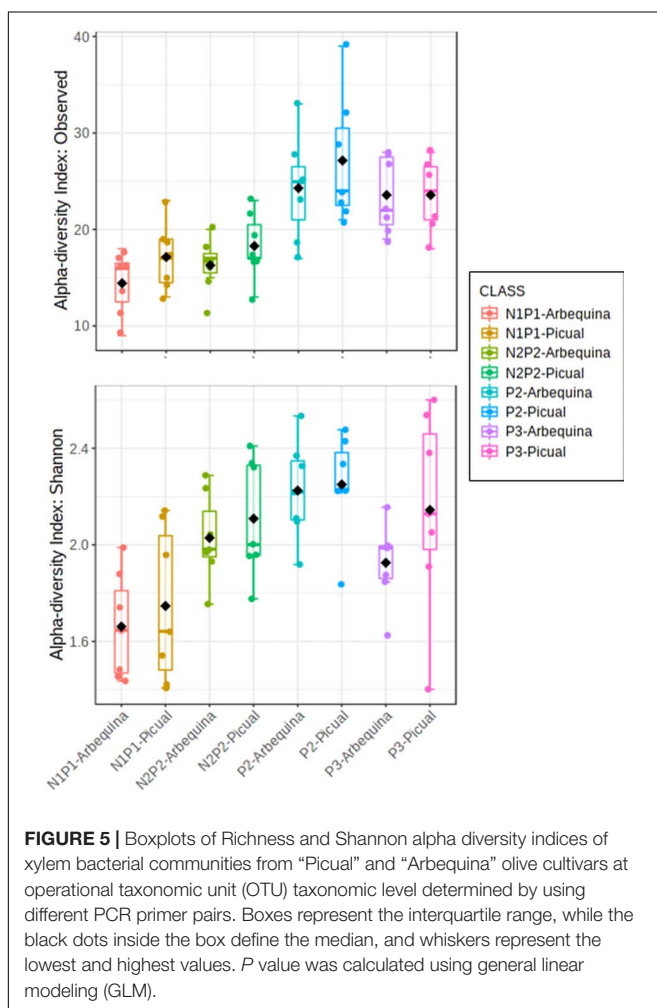
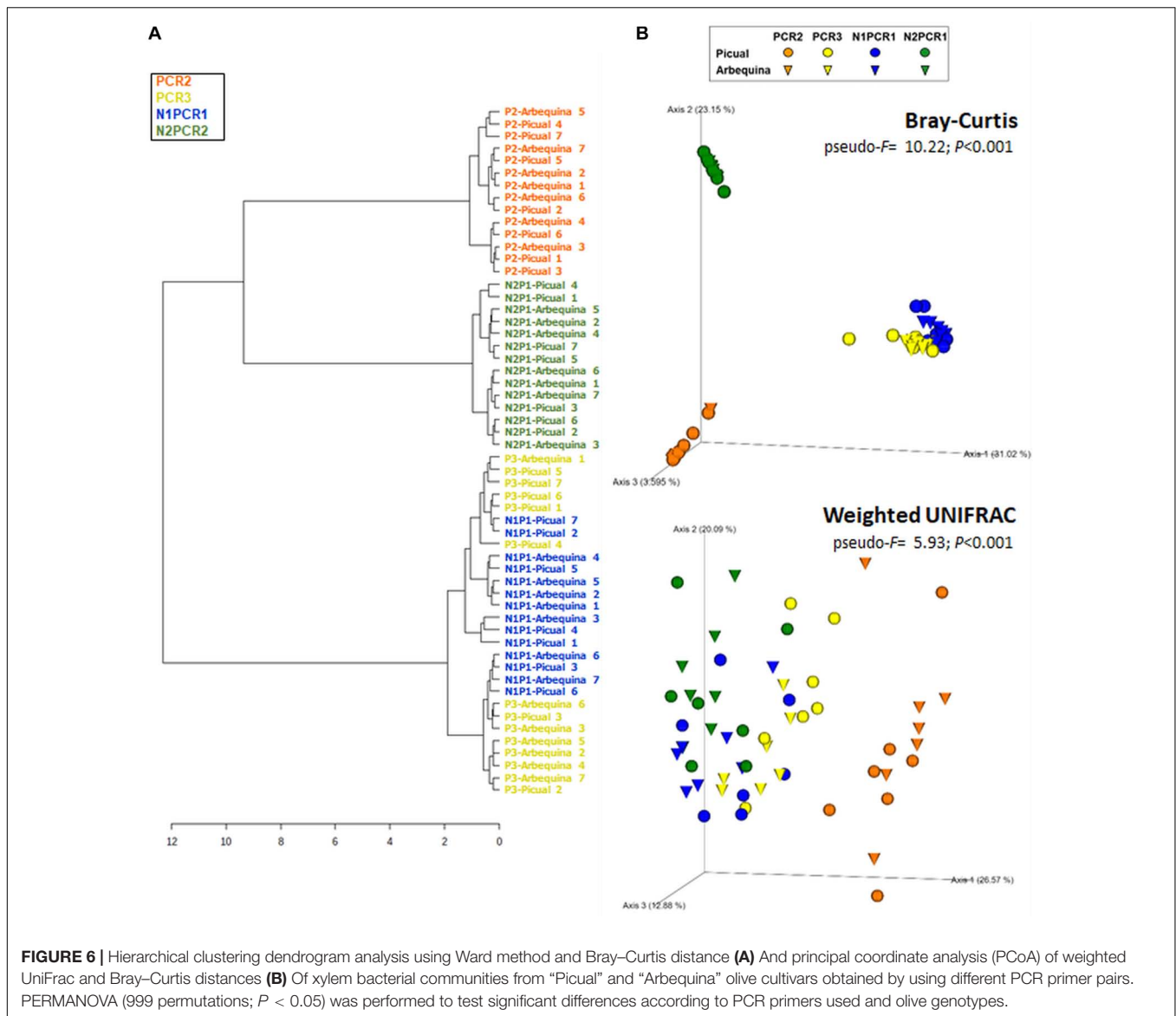


FIGURE 5 | Boxplots of Richness and Shannon alpha diversity indices of xylem bacterial communities from “Picual” and “Arbequina” olive cultivars at operational taxonomic unit (OTU) taxonomic level determined by using different PCR primer pairs. Boxes represent the interquartile range, while the black dots inside the box define the median, and whiskers represent the lowest and highest values. P value was calculated using general linear modeling (GLM).



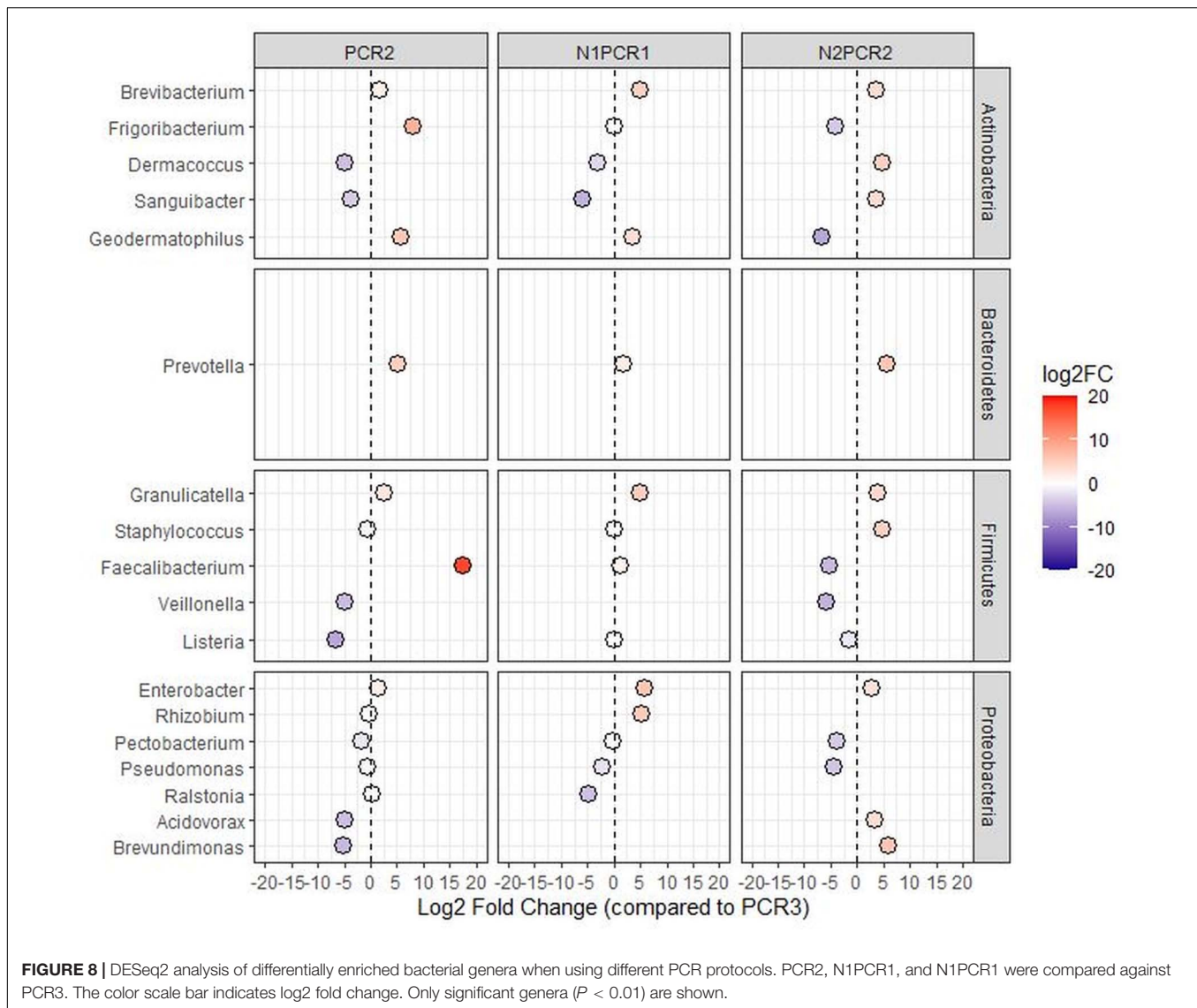
(mean frequency ranged between 42.4 and 3.8%, in that order) **(Figure 7 and Supplementary Figure 3)**.

Finally, at the genus level, 50 genera comprised the core bacteria, where the highest numbers of genera were detected by direct PCR primers (154 in PCR2 and 162 in PCR3), while the lowest numbers were obtained for both nested PCR protocols (100 in N1PCR1 and 107 in N2PCR1). Direct PCR protocols showed the same unique number of genera (51 each one), while N2PCR1 displayed nine unique genera. No unique genera were found in N1PCR1 **(Table 4 and Supplementary Figure 3)**. Differential abundance analysis using DESeq2 displayed significant differences among genera between each of the PCR primer pairs tested when compared to the selected PCR3 protocol. Thus, DESeq2 identified a significant and high enrichment (Log_2 Fold Change > 5) of *Faecalibacterium*, *Prevotella*, *Geodermatophilus*, and *Frigoribacterium* in PCR2, *Rhizobium*, *Enterobacter*, *Granulicatella*, and *Brevibacterium* in

N1PCR1, and *Brevundimonas*, *Staphylococcus*, *Prevotella*, and *Dermacoccus* in N2PCR2 **(Figure 8)**. Interestingly, *Enterobacter*, *Granulicatella*, *Prevotella*, and *Brevibacterium* presented a significant higher abundance in all PCR protocols when comparing to PCR3, while *Pseudomonas* and *Pectobacterium* displayed an opposite behavior **(Figure 8)**.

DISCUSSION

Although assessment and optimization of different DNA extraction protocols should be one of the most important initial steps when developing a protocol for analysis of microbial communities in a new plant niche due to its potential significant influence on the structure and diversity of the recovered community profile (Rubin et al., 2014), for all the studies assessing olive microbiota, this assessment is lacking.



or the inclusion of a preheating treatment (Groups 2 and 3) that can affect differentially bacterial cell wall lysis (Teng et al., 2018). After performing each DNA extraction protocol, we showed that the different DNA extraction methods led to various levels of DNA quantity and quality. However, higher DNA yields were not necessarily correlated with a better amplification efficiency (Table 1). From the 12 DNA kits assayed, we found PowerPlant as the most suitable DNA extraction kit for the characterization of olive xylem sap microbiota due to the quality and concentration of DNA obtained, it is a short time-consuming procedure, the market price is within the lowest, and the high percentage (92%) of sequences assigned to bacteria. These results are in line with other works that support its usefulness for subsequent microbiota analysis (Corcoll et al., 2017). However, PowerSoil kit also showed good characteristics as those shown by the PowerPlant kit, although the time for extraction is a little bit longer. Consequently, it could also be considered useful for

the analysis of olive microbiota, especially on studies in which other plant niches such as the rhizosphere may also be explored. In fact, this kit has gained special interest as the standard technique for extracting microbial DNA from environmental samples, including two of the largest microbiome initiatives, the Earth Microbiome Project and the Human Microbiome Project (Rubin et al., 2014).

Since bacterial 16S rRNA gene sequences present a high homology with chloroplast and mitochondrial rRNA genes (Sakai et al., 2004), the discrimination between host plant DNA, organellar rDNA, and microbial 16S rDNA suppose an enormous challenge for the application of PCR-based methods in the study of plant microbiota (Beckers et al., 2016; Jackrel et al., 2017). Therefore, we determined the effect of primer pairs on xylem sap bacterial community characterization when using a mock- and an indigenous-microbial community extracted from two olive cultivars. The primers selected for our study had been reported to reduce co-amplification or

organellar sequences being widely and commonly used for the analysis of plant-associated bacterial communities using Illumina MiSeq sequencing. The ZymoBIOMICS Microbial Community Standard, designed to assess bias and errors in DNA extraction protocols and to improve the quality and reproducibility of metagenomics analyses, was used to determine the efficacy of four primer pairs targeting different hypervariable regions of 16S rRNA gene for giving an accurate representation of the xylem microbial communities. This validation procedure has been used by other authors (e.g., McGovern et al., 2018; Neuberger-Castillo et al., 2020). The ZymoBIOMICS Microbial Community Standard offers a well-defined composition and an accurately characterized mock community consisting of three Gram-negative and five Gram-positive bacteria, easy and tough to lyse, respectively, and two tough-to-lyse yeasts with varying sizes and cell wall composition. This wide range of organisms with different properties enables characterization, optimization, and validation of different lysis methods and PCR amplification protocols, and it can guide construction of entire workflows being used as a routine quality control. This mock microbial DNA community standard allowed the identification of significant differences among the different primer pairs used in the study, with PCR3 (799F/1193R) as the most accurate primer pair in combination with the Silva 132 database for taxonomic assignment when comparing results with the theoretical composition of ZymoBIOMICS (Figure 3). A significant influence of the choice of primer pairs using mock communities has also been shown in other studies (Fouhy et al., 2015; Teng et al., 2018). These results emphasize the need for testing several primers to use standardized approaches to analyze microbial communities, especially when exploring new plant niches.

When using natural olive samples from “Arbequina” and “Picual” cultivars and comparing the four primer pairs, results indicated that primer pair 799F-1193R recovered the highest number of bacterial OTUs (242) and displayed a low co-amplification rate of organellar rRNA gene, although for several of the samples, a removal of unspecific bands by agarose gel purification was needed prior to library sequencing. The Actinobacteria, Firmicutes, and Proteobacteria phyla have been described as the most abundant in olive xylem sap by other authors (Sofa et al., 2019; Anguita-Maeso et al., 2020). In our study, although the same most abundant phyla were detected by all PCRs, some phyla were detected exclusively by some PCR primers. For instance, PCR2 was the only one detecting Dependenteae, Verrucomicrobia, Nitrospirae, Planctomycetes, and Cyanobacteria, whereas the Armatimonadetes and Patescibacteria phyla were exclusively detected by PCR3. At lower taxonomic level, 48 families formed the core microbiota among all PCR primer pairs tested. Among these, the families Propionibacteriaceae, Staphylococcaceae, Sphingomonadaceae, Burkholderiaceae, Enterobacteriaceae, and Pseudomonadaceae were the most predominant, agreeing with the results obtained in olive by other authors (Müller et al., 2015; Fausto et al., 2018; Sofa et al., 2019; Anguita-Maeso et al., 2020; Giampetrucchi et al., 2020). However, the

relative abundance of each family varied according to the PCR primer pairs used. Thus, PCR3 and N1PCR1 detected higher abundance of Propionibacteriaceae in comparison with PCR2 and N2PCR1. On the other hand, the families Staphylococcaceae and Enterobacteriaceae were detected at higher frequencies with PCR2, whereas the relative amount of Sphingomonadaceae was higher when using N2PCR1. Similarly, the families Burkholderiaceae and Pseudomonadaceae presented higher relative abundance when using nested PCR primers instead of direct PCRs. These differences obtained in bacterial community structure depending on the target primer set used may be due to the different hypervariable regions of 16S rRNA gene targeted (Table 2) or a disparity of matching efficiencies between primers for some microbial groups as has been found in other studies (Cruaud et al., 2014). The differences obtained in the nested PCRs as compared to the direct PCR approaches can be due to the introduction of a bias during amplification for the most abundant bacterial families, as it has been recently shown (Yu et al., 2015). Consequently, this approach should be used only when the amount of DNA is too low to be amplified by standard PCR to avoid potential biases measuring the bacterial community structure.

The tendency to find a low number of taxa in nested PCRs when compared to direct PCR protocols was also observed at the genus level. Although 50 genera composed the core bacterial microbiota, we found different unique genera depending on the PCR protocol. Interestingly, a high number of unique genera were found in PCR2 and PCR3 (51 each one), whereas N2PCR1 showed nine exclusive genera and no one was found unique in N1PCR1. Differential abundance analysis showed distinct enrichment of some genera based on the PCR primer pairs used. In such a way, when comparing PCR3 against the other three PCR protocols, we observed a significant enrichment of *Faecalibacterium*, *Prevotella*, *Geodermatophilus*, and *Frigoribacterium* in PCR2; *Rhizobium*, *Enterobacter*, *Granulicatella*, and *Brevibacterium* in N1PCR1, and *Brevundimonas*, *Staphylococcus*, *Prevotella*, and *Dermaococcus* in N2PCR2. Within this comparison, *Faecalibacterium*, *Enterobacter*, and *Brevundimonas* in PCR2, N1PCR1, and N2PCR2, respectively, displayed the greatest values of enrichment. These genera have been detected in other plant niches previously such as in rhizosphere or phyllosphere (Teixeira et al., 2010; Compant et al., 2019). Among them, *Brevundimonas* has been already described to confer fitness advantages to host plants due to its potential to act as a soil bioremediator and plant growth promoter (Kumar and Gera, 2014; Singh et al., 2016), although its use as a biological control agent against plant diseases is compromised due to the human pathogenic activity presented by some members of this genus (Ryan and Pembroke, 2018). On the other hand, we observed differences in “Picual” and “Arbequina” olive genotypes where the phyla *Acidobacteria* and *Gemmatimonadetes* were only present in “Picual” cultivar. However, an in-depth study targeting a wide range of olive cultivars is needed to better understand the effect of olive genotype in shaping the xylem microbiome.

Our study demonstrates significant and noticeable differences among DNA extraction kits and PCR primers that influence

the interpretation of the bacterial community composition of olive xylem sap. Overall, our findings provide new insights and an integrated assessment of both the benefits and drawbacks of several commercially available DNA extraction kits and offer guidance to other researchers in the choice of best-suited kits, considering cell lysis efficacy, DNA yield, microbial diversity recovered, processing time, and cost-effectiveness. Also, this study highlights the crucial choice of a good primer set to provide a non-biased vision of the true composition of the analyzed microbial community avoiding the co-amplification of plant organellar rRNA genes. Our results offered the road map to design an optimized strategy for selecting the most suitable PCR primer pair for assessing bacterial communities based on the use of an artificial commercially available mock community together with a precise and accurate bioinformatic workflow that can be followed when optimizing protocols for accurate depiction of the bacterial communities present in xylem vessels or other plant niches.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>, PRJNA684121.

AUTHOR CONTRIBUTIONS

CH, MA-M, and BL conceived the research, performed statistical and bioinformatic analyses, interpreted the results, and wrote the manuscript. CH and MA-M prepared the materials and equipment and performed the experiments. MM and JN-C contributed to reviewing the manuscript and interpreted the results. All authors viewed the draft of the manuscript.

REFERENCES

- Anguita-Maeso, M., Olivares-García, C., Haro, C., Imperial, J., Navas-Cortés, J. A., and Landa, B. B. (2020). Culture-dependent and culture-independent characterization of the olive xylem microbiota: effect of sap extraction methods. *Front. Plant Sci.* 10:1708. doi: 10.3389/fpls.2019.01708
- Aranda, S., Montes-Borrego, M., Jiménez-Díaz, R. M., and Landa, B. B. (2011). Microbial communities associated with the root system of wild olives (*Olea europaea* L. subsp. *europaea* var. *sylvestris*) are good reservoirs of bacteria with antagonistic potential against *Verticillium dahliae*. *Plant Soil* 343, 329–345. doi: 10.1007/s11104-011-0721-2
- Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 1:110674. doi: 10.18637/jss.v067.i01
- Beals, E. W. (1984). Bray-Curtis ordination: an effective strategy for analysis of multivariate ecological data. *Adv. Ecol. Res.* 14, 1–55. doi: 10.1016/S0065-2504(08)60168-3
- Beckers, B., Op De Beeck, M., Thijs, S., Truyens, S., Weyens, N., Boerjan, W. et al. (2016). Performance of 16S rDNA primer pairs in the study of rhizosphere and endosphere bacterial microbiomes in metabarcoding studies. *Front. Microbiol.* 7:650. doi: 10.3389/fmicb.2016.00650
- Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R.*

FUNDING

This study was funded by the project AGL2016-75606-R (Programa Estatal de I+D Orientado a los Retos de la Sociedad from Spanish Government, the Spanish State Research Agency, and FEDER-EU) and Project XF-ACTORS (grant 727987) from the European Union's Horizon 2020 Framework Research Programme. MA-M is a recipient of a research fellowship BES-2017-082361 from the Spanish Ministry of Economy and Competitiveness.

ACKNOWLEDGMENTS

We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2021.640829/full#supplementary-material>

Supplementary Figure 1 | Richness rarefaction curves at OTU taxonomic level obtained when using different DNA extraction kits and after taxonomic assignments with the Greengenes_13-8 and Silva_132 databases. Error bars show the standard deviation.

Supplementary Figure 2 | Prevalence Venn diagram showing the unique and shared bacterial taxa at phylum, class, order, family, and genera level using the four clustered DNA extraction kits obtained shown in **Figure 2**. For each taxa, the venn diagram is shown using the Greengenes 13-8 and Silva_132 databases. Tables show the bacterial taxonomy interaction within each reference database.

Supplementary Figure 3 | Prevalence Venn diagram showing the unique and shared bacterial taxa obtained at phylum, class, order, family, and genera level using the four PCR protocols. Tables show the bacterial taxonomy interaction within each PCR protocol.

- Stat. Soc. Ser. B* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x
- Berg, G., Grube, M., Schloter, M., and Smalla, K. (2014). Unraveling the plant microbiome: looking back and future perspectives. *Front. Microbiol.* 5:148. doi: 10.3389/fmicb.2014.00148
- Berg, G., Rybakova, D., Grube, M., and Köberl, M. (2016). The plant microbiome explored: implications for experimental botany. *J. Exp. Bot.* 67, 995–1002. doi: 10.1093/jxb/erv466
- Bodenhausen, N., Horton, M. W., and Bergelson, J. (2013). Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. *PLoS One* 8:e56329. doi: 10.1371/journal.pone.0056329
- Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C., Al-Ghalith, G. A., et al. (2018). QIIME 2: reproducible, interactive, scalable, and extensible microbiome data science. *PeerJ Prepr.* 6:e27295v2. doi: 10.7287/peerj.preprints.27295v2
- Brooks, J. P., Edwards, D. J., Harwich, M. D., Rivera, M. C., Fettweis, J. M., Serrano, M. G., et al. (2015). The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiol.* 15:66. doi: 10.1186/s12866-015-0351-6
- Burbach, K., Seifert, J., Pieper, D. H., and Camarinha-Silva, A. (2016). Evaluation of DNA extraction kits and phylogenetic diversity of the porcine

- gastrointestinal tract based on Illumina sequencing of two hypervariable regions. *Microbiologyopen* 5, 70–82. doi: 10.1002/mbo3.312
- Caliz, J., Montes-Borrego, M., Triadó-Margarit, X., Metsis, M., Landa, B. B., and Casamayor, E. O. (2015). Influence of edaphic, climatic, and agronomic factors on the composition and abundance of nitrifying microorganisms in the rhizosphere of commercial olive crops. *PLoS One* 10:e0125787. doi: 10.1371/journal.pone.0125787
- Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7:335. doi: 10.1038/nmeth.f.303
- Chelius, M. K., and Triplett, E. W. (2001). The diversity of archaea and bacteria in association with the roots of *Zea mays* L. *Microb. Ecol.* 41, 252–263. doi: 10.1007/s002480000087
- Chong, J., Liu, P., Zhou, G., and Xia, J. (2020). Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat. Protoc.* 15, 799–821. doi: 10.1038/s41596-019-0264-1
- Claesson, M. J., Wang, Q., O'Sullivan, O., Greene-Diniz, R., Cole, J. R., Ross, R. P., et al. (2010). Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Res.* 38:e00200. doi: 10.1093/nar/gkq873
- Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: ecology, functions, and emerging trends in microbial application. *J. Adv. Res.* 19, 29–37. doi: 10.1016/j.jare.2019.03.004
- Corcoll, N., Österlund, T., Sinclair, L., Eiler, A., Kristiansson, E., Backhaus, T., et al. (2017). Comparison of four DNA extraction methods for comprehensive assessment of 16S rRNA bacterial diversity in marine biofilms using high-throughput sequencing. *FEMS Microbiol. Lett.* 364:fnx139. doi: 10.1093/femsle/fnx139
- Craud, P., Vigneron, A., Lucchetti-Miganeh, C., Ciron, P. E., Godfroy, A., and Cambon-Bonavita, M.-A. (2014). Influence of DNA extraction method, 16S rRNA targeted hypervariable regions, and sample origin on microbial diversity detected by 454 pyrosequencing in marine chemosynthetic ecosystems. *Appl. Environ. Microbiol.* 80, 4626–4639. doi: 10.1128/AEM.00592-14
- DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05
- Dos Santos, M., Kavamura, V., Reynaldo, E., Souza, D., Da Silva, E., and May, A. (2017). Bacterial structure of agricultural soils with high and low yields. *J. Plant Pathol. Microbiol.* 8:405. doi: 10.4172/2157-7471.1000405
- Fausto, C., Mininni, A. N., Sofo, A., Crecchio, C., Scagliola, M., Dichio, B., et al. (2018). Olive orchard microbiome: characterisation of bacterial communities in soil-plant compartments and their comparison between sustainable and conventional soil management systems. *Plant Ecol. Divers.* 11, 597–610. doi: 10.1080/17550874.2019.1596172
- Feehery, G. R., Yigit, E., Oyola, S. O., Langhorst, B. W., Schmidt, V. T., Stewart, F. J., et al. (2013). A method for selectively enriching microbial DNA from contaminating vertebrate host DNA. *PLoS One* 8:e76096. doi: 10.1371/journal.pone.0076096
- Fernández-González, A. J., Cardoni, M., Gómez-Lama Cabanás, C., Valverde-Corredor, A., Villadas, P. J., Fernández-López, M., et al. (2020). Linking belowground microbial network changes to different tolerance level towards *Verticillium* wilt of olive. *Microbiome* 8:11. doi: 10.1186/s40168-020-0787-2
- Fouhy, F., Deane, J., Rea, M. C., O'Sullivan, L., Ó Ross, R. P., O'Callaghan, G., et al. (2015). The effects of freezing on faecal microbiota as determined using MiSeq sequencing and culture-based investigations. *PLoS One* 10:e0119355. doi: 10.1371/journal.pone.0119355
- Ghyselinck, J., Pfeiffer, S., Heylen, K., Sessitsch, A., and De Vos, P. (2013). The effect of primer choice and short read sequences on the outcome of 16S rRNA gene based diversity studies. *PLoS One* 8:e71360. doi: 10.1371/journal.pone.0071360
- Giampetruzzi, A., Baptista, P., Morelli, M., Cameirão, C., Lino Neto, T., Costa, D., et al. (2020). Differences in the endophytic microbiome of olive cultivars infected by *Xylella fastidiosa* across seasons. *Pathogens* 9:723. doi: 10.3390/pathogens9090723
- Gómez-Lama Cabanás, C., Ruano-Rosa, D., Legarda, G., Pizarro-Tobías, P., Valverde-Corredor, A., Triviño, J. C., et al. (2018). *Bacillales* members from the olive rhizosphere are effective biological control agents against the defoliating pathotype of *Verticillium dahliae*. *Agriculture* 8:90. doi: 10.3390/agriculture8070090
- Green, S. J., and Minz, D. (2005). Suicide polymerase endonuclease restriction, a novel technique for enhancing PCR amplification of minor DNA templates. *Appl. Environ. Microbiol.* 71, 4721–4727. doi: 10.1128/AEM.71.8.4721-4727.2005
- Hacquard, S., and Schadt, C. W. (2015). Towards a holistic understanding of the beneficial interactions across the *Populus* microbiome. *New Phytol.* 205, 1424–1430. doi: 10.1111/nph.13133
- Henderson, G., Cox, F., Kittelmann, S., Miri, V. H., Zethof, M., Noel, S. J., et al. (2013). Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. *PLoS One* 8:e74787. doi: 10.1371/journal.pone.0074787
- Jackrel, S. L., Owens, S. M., Gilbert, J. A., and Pfister, C. A. (2017). Identifying the plant-associated microbiome across aquatic and terrestrial environments: the effects of amplification method on taxa discovery. *Mol. Ecol. Resour.* 17, 931–942. doi: 10.1111/1755-0998.12645
- Jiménez-Díaz, R. M., Cirulli, M., Bubici, G., del Mar Jiménez-Gasco, M., Antoniou, P. P., Tjamos, E. C. et al. (2011). *Verticillium* Wilt, a major threat to olive production: current status and future prospects for its management. *Plant Dis.* 96, 304–329. doi: 10.1094/PDIS-06-11-0496
- Kennedy, N. A., Walker, A. W., Berry, S. H., Duncan, S. H., Farquarson, F. M., Louis, P., et al. (2014). The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16S rRNA gene sequencing. *PLoS One* 9:e88982. doi: 10.1371/journal.pone.0088982
- Keykhasaber, M., Pham, K. T. K., Thomma, B. P. H. J., and Hiemstra, J. A. (2017). Reliable detection of unevenly distributed *Verticillium dahliae* in diseased olive trees. *Plant Pathol.* 66, 641–650. doi: 10.1111/ppa.12647
- Knudsen, B. E., Bergmark, L., Munk, P., Lukjancenko, O., Priemé, A., Aarestrup, F. M., et al. (2016). Impact of sample type and DNA isolation procedure on genomic inference of microbiome composition. *mSystems* 1:e00095-16. doi: 10.1128/mSystems.00095-16
- Kumar, V., and Gera, R. (2014). Isolation of a multi-trait plant growth promoting *Brevundimonas* sp. and its effect on the growth of Bt-cotton. *3 Biotech.* 4, 97–101. doi: 10.1007/s13205-013-0126-4
- Landa, B. B., Montes-Borrego, M., Aranda, S., Soriano, M. A., Gómez, J. A., and Navas-Cortés, J. A. (2014). Soil factors involved in the diversity and structure of soil bacterial communities in commercial organic olive orchards in Southern Spain. *Environ. Microbiol. Rep.* 6, 196–207. doi: 10.1111/1758-2229.12148
- Landa, B. B., Pérez, A. G., Luaces, P., Montes-Borrego, M., Navas-Cortés, J. A., and Sanz, C. (2019). Insights into the effect of *Verticillium dahliae* defoliating-pathotype infection on the content of phenolic and volatile compounds related to the sensory properties of virgin olive oil. *Front. Plant Sci.* 10:232. doi: 10.3389/fpls.2019.00232
- Lei, S., Xu, X., Cheng, Z., Xiong, J., Ma, R., Zhang, L., et al. (2019). Analysis of the community composition and bacterial diversity of the rhizosphere microbiome across different plant taxa. *Microbiologyopen* 8:e00762. doi: 10.1002/mbo3.762
- Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8
- Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005
- McCully, M. E. (2001). Niches for bacterial endophytes in crop plants: a plant biologist's view. *Funct. Plant Biol.* 28, 983–990. doi: 10.1071/PP01101
- McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., et al. (2011). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6:610. doi: 10.1038/ismej.2011.139
- McGovern, E., Waters, S. M., Blackshields, G., and McCabe, M. S. (2018). Evaluating established methods for rumen 16S rRNA amplicon sequencing with mock microbial populations. *Front. Microbiol.* 9:1365. doi: 10.3389/fmicb.2018.01365

- Mercado-Blanco, J., Rodríguez-Jurado, D., Hervás, A., and Jiménez-Driaz, R. M. (2004). Suppression of *Verticillium* wilt in olive planting stocks by root-associated fluorescent. *Biol. Control* 30, 474–486. doi: 10.1016/j.biocontrol.2004.02.002
- Montes-Borrego, M., Navas-Cortés, J. A., and Landa, B. B. (2013). Linking microbial functional diversity of olive rhizosphere soil to management systems in commercial orchards in southern Spain. *Agric. Ecosyst. Environ.* 181, 169–178. doi: 10.1016/j.agee.2013.09.021
- Müller, H., Berg, C., Landa, B. B., Auerbach, A., Moissl-Eichinger, C., and Berg, G. (2015). Plant genotype-specific archaeal and bacterial endophytes but similar *Bacillus* antagonists colonize Mediterranean olive trees. *Front. Microbiol.* 6:138. doi: 10.3389/fmicb.2015.00138
- Neuberger-Castillo, L., Hamot, G., Marchese, M., Sanchez, I., Ammerlaan, W., and Betsou, F. (2020). Method validation for extraction of DNA from human stool samples for downstream microbiome analysis. *Biopreserv. Biobank.* 18, 102–116. doi: 10.1089/bio.2019.0112
- Porras-Alfaro, A., and Bayman, P. (2011). Hidden fungi, emergent properties: endophytes and microbiomes. *Annu. Rev. Phytopathol.* 49, 291–315. doi: 10.1146/annurev-phyto-080508-081831
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, 590–596. doi: 10.1093/nar/gks1219
- Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahe, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584. doi: 10.7717/peerj.2584
- Rubin, B. E. R., Sanders, J. G., Hampton-Marcell, J., Owens, S. M., Gilbert, J. A., and Moreau, C. S. (2014). DNA extraction protocols cause differences in 16S rRNA amplicon sequencing efficiency but not in community profile composition or structure. *Microbiologyopen* 3, 910–921. doi: 10.1002/mbo3.216
- Ryan, M. P., and Pembroke, J. T. (2018). *Brevundimonas* spp: emerging global opportunistic pathogens. *Virulence* 9, 480–493. doi: 10.1080/21505594.2017.1419116
- Sakai, M., Matsuka, A., Komura, T., and Kanazawa, S. (2004). Application of a new PCR primer for terminal restriction fragment length polymorphism analysis of the bacterial communities in plant roots. *J. Microbiol. Methods* 59, 81–89. doi: 10.1016/j.mimet.2004.06.005
- Santoyo, G., Moreno-Hagelsieb, G., Orozco-Mosqueda, M., Del, C., and Glick, B. R. (2016). Plant growth-promoting bacterial endophytes. *Microbiol. Res.* 183, 92–99. doi: 10.1016/j.micres.2015.11.008
- Saponari, M., Giampetruzzi, A., Loconsole, G., Boscia, D., and Saldarelli, P. (2018). *Xylella fastidiosa* in olive in Apulia: where we stand. *Phytopathology* 109, 175–186. doi: 10.1094/PHYTO-08-18-0319-FI
- Singh, N., Marwa, N., Mishra, S. K., Mishra, J., Verma, P. C., Rathaur, S., et al. (2016). *Brevundimonas diminuta* mediated alleviation of arsenic toxicity and plant growth promotion in *Oryza sativa* L. *Ecotoxicol. Environ. Saf.* 125, 25–34. doi: 10.1016/j.ecoenv.2015.11.020
- Sofa, A., Mininni, A. N., Fausto, C., Scagliola, M., Crecchio, C., Xiloyannis, C., et al. (2019). Evaluation of the possible persistence of potential human pathogenic bacteria in olive orchards irrigated with treated urban wastewater. *Sci. Total Environ.* 658, 763–767. doi: 10.1016/j.scitotenv.2018.12.264
- Sogin, M. L., Morrison, H. G., Huber, J. A., Mark Welch, D., Huse, S. M., Neal, P. R., et al. (2006). Microbial diversity in the deep sea and the underexplored “rare biosphere”. *Proc. Natl. Acad. Sci. U.S.A.* 103, 12115–12120. doi: 10.1073/pnas.0605127103
- Stulberg, E., Fravel, D., Proctor, L. M., Murray, D. M., LoTempio, J., Chrisey, L., et al. (2016). An assessment of US microbiome research. *Nat. Microbiol.* 1:15015. doi: 10.1038/nmicrobiol.2015.15
- Teixeira, L. C. R. S., Peixoto, R. S., Cury, J. C., Sul, W. J., Pellizari, V. H., Tiedje, J., et al. (2010). Bacterial diversity in rhizosphere soil from Antarctic vascular plants of Admiralty Bay, maritime Antarctica. *ISME J.* 4, 989–1001. doi: 10.1038/ismej.2010.35
- Teng, F., Darveekaran Nair, S. S., Zhu, P., Li, S., Huang, S., Li, X., et al. (2018). Impact of DNA extraction method and targeted 16S-rRNA hypervariable region on oral microbiota profiling. *Sci. Rep.* 8:16321. doi: 10.1038/s41598-018-34294-x
- Vergine, M., Meyer, J. B., Cardinale, M., Sabella, E., Hartmann, M., Cherubini, P., et al. (2020). The *Xylella fastidiosa*-resistant olive cultivar “Leccino” has stable endophytic microbiota during the olive quick decline syndrome (OQDS). *Pathogens* 9:35. doi: 10.3390/pathogens9010035
- Walker, J. J., and Pace, N. R. (2007). Phylogenetic composition of rocky mountain endolithic microbial ecosystems. *Appl. Environ. Microbiol.* 73, 3497–3504. doi: 10.1128/AEM.02656-06
- Wasimuddin, Schlaeppi, K., Ronchi, F., Leib, S. L., Erb, M., and Ramette, A. (2020). Evaluation of primer pairs for microbiome profiling from soils to humans within the One Health framework. *Mol. Ecol. Resour.* 20, 1558–1571. doi: 10.1111/1755-0998.13215
- Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., et al. (2014). The SILVA and “All-species Living Tree Project (LTP)” taxonomic frameworks. *Nucleic Acids Res.* 42, 643–648. doi: 10.1093/nar/gkt1209
- Yu, G., Fadrosh, D., Goedert, J. J., Ravel, J., and Goldstein, A. M. (2015). Nested PCR biases in interpreting microbial community structure in 16S rRNA gene sequence datasets. *PLoS One* 10:e0132253. doi: 10.1371/journal.pone.0132253

Conflict of Interest: MM was employed by the company Testsystems LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Haro, Anguita-Maeso, Metsis, Navas-Cortés and Landa. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.