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Impact of DNA purification method and primer selection on 16S rRNA gene metabarcoding on wine

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ABSTRACT

Aim: The high-throughput sequencing methods have revolutionized the study of the microbiota in different matrices including those of the grapevine production chain. DNA extraction is a crucial step in the sample processing. In this study, we compared different DNA purification methods and two primer sets for 16S rRNA gene metabarcoding to evaluate the best protocol to explore the wine microbiota by metabarcoding.
Methods and results: We collected a wine from Barbera grapes after malolactic fermentation previously inoculated by *Oenococcus oeni* starter. The same sample was used to evaluate the best performing protocol to study the wine microbiota. DNA was purified using nine different methods and then amplified for the 16S rRNA gene with two primer sets (according to Illumina or Earth Microbiome Project protocols). The obtained amplicons were then sequenced in a single sequencing session on an Illumina MiSeq. We evaluated the best protocol considering DNA concentration and purity, alpha (Observed species) and beta diversity from metabarcoding analysis.
The sequencing generated 36,031,756 reads in total. Although no statistically significant difference was observed between purification methods or primer sets, better results were obtained with phenol-chloroform DNA purification combined to Earth Microbiome Project primers.
Metabarcoding was able to highlight the domination of the inoculum, *O. oeni*, representing the main species of the analyzed wine microbiota.
Conclusion: Our data show that, for the tested wine, metabarcoding output is more influenced by the primer set than by the DNA purification method. Moreover, the metabarcoding detected that *O. oeni* represents the main species, evidencing the domination of the inoculum done with lyophilized commercial preparation of this species. Other lactic acid bacteria are present at a much lower abundance.
Significance and impact of the study: This is the first report applying the 16S rRNA gene metabarcoding to study the microbiota of wine. For this reason, the evaluation of alternative methods for DNA processing is essential for future research using this innovative methodology.

KEYWORDS

wine microbiota wine DNA purification, 16S rRNA gene metabarcoding, wine NGS, wine microbial community

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INTRODUCTION

The high-throughput sequencing methods have revolutionized the study of the microbiota in different matrices including those of the grapevine production chain (Franzosa *et al.*, 2015; Stefanini and Cavalieri, 2018). In recent years, numerous studies have been proposed concerning the description of the microbiological profile both from the bacterial and the fungi point of view (see review of Morgan *et al.*, 2017). With this latest sequencing technique, DNA extraction is a crucial step in the sample processing (Knudsen *et al.*, 2016) and several methods have been proposed to extract DNA from the wine matrix (Belda *et al.*, 2017). The aim of this study was to test different extraction protocols, both in-house methodology and commercial kits, to verify their suitability for subsequent 16S metabarcoding. In addition, two primer sets were also evaluated.

MATERIALS AND METHODS

1. Wine DNA extraction

To standardize the matrix from which to test different DNA extraction methods, we collected a wine from Barbera grapes, inoculated with *Oenococcus oeni*, after malolactic fermentation. Aliquots of 1 ml of raw wine with lees were stored in 1.5 ml tubes, centrifuged at 10,000 RCF for 2 min, washed with 1 ml of U.P. sterile H_20 and centrifuged at 10,000 RCF for 2 min; supernatant was discarded, and pellet stored at -80 °C.

DNA extraction was performed according to nine different methods in at least two replicates, summarized in Table 1.

2. Performance evaluation

DNA concentration, purity and integrity were evaluated by fluorimetry (Qubit dsDNA HS kit - Thermo Fisher Scientific, Waltham, MA, USA), spectrophotometry (Vivaspec LS, Sartorius, Göttingen, Germany), and micro-capillary based electrophoresis (DNA 12000 and DNA HS chips on BioAnalyzer 2100, Agilent Technologies, Santa Clara, CA, USA), respectively.

The Illumina 16S Metagenomic Sequencing Library Preparation was adapted to the NEBNext® Q5 Hot Start HiFi PCR Master Mix (New England BioLabs, Cambridge, MA, USA) with the following thermal profile: 98 °C x 30 sec, 25 x [98 °C x 10 sec, 55 °C x 30 sec, 65 °C x 45 sec], 65 °C x 5 min.

We used two different 16S rRNA gene primer sets for a comparison: the primer set suggested by Illumina, targeting ~420bp of the V3-V4 regions (Klindworth *et al.*, 2013) (hereafter ILL), and the 515FB/806RB primers from the Earth Microbiome Project (Caporaso *et al.*, 2011), targeting ~250bp of the V4 region (hereafter EMP). For each primer set, a blank PCR control was used and processed as a sample.

A total of 38 PCR products (18 samples and 1 negative PCR control – NTC – for each primer set) were sequenced in a single run (2 x 250 bp paired-end) on an Illumina MiSeq platform. Products of the first PCR were observed on 2 % agarose gel electrophoresis to check the amplification.

The raw data were analyzed with the DADA2 pipeline within the R framework, including quality check, error rate estimation, forward/reverse reads merge, chimera removal, Amplicon Sequence Variants (ASVs, equivalent to OTUs) determination, and taxa assignment to the RDP Training Set 14 (Callahan *et al.*, 2016). Alpha and beta diversities and their statistical support were estimated with *phyloseq* and *vegan* packages (McMurdie and Holmes, 2013; Oksanen *et al.*, 2017). In details, the "observed species" index was used to describe the alpha diversity, while the Bray Curtis dissimilarity (accounting only the abundance information) was used to obtain the matrix for the beta diversity and the Principal Coordinate Analysis (PCoA). Statistical significance was tested with Kruskal-Wallis test for alpha diversity and with *adonis* test for beta diversity.

RESULTS

DNA concentration and purity obtained from the wine aliquots using the different purification methods are reported in Table 1. Based on spectrophotometry, the tested methods returned a large range of values in terms of quantity and purity. Methods C, D and E returned the best DNA purity values, as well as the highest yield (together with method B). In general, results were consistent within method, despite the small replicate number. Fluorimetry returned much lower concentration values, ranging from 0.24 to 7.03 ng/µl. Again, methods D and E returned the highest yield values.

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TABLE 1. Sample list with DNA purification methods, concentration and purity.

Sample number	DNA purification method (code)	Spectrophotometer			Qubit dsDNA HS
		DNA concentration (ng/µl)	260/280	260/230	DNA concentration (ng/µl)
1	5Prime ArchivePure DNA Yeast & gram-+ KIT [A]	145.5	1.76	0.57	1.33
2	5Prime ArchivePure DNA Yeast & gram-+ KIT [A]	132	1.77	0.61	1.16
5	5Prime ArchivePure DNA Yeast & gram-+ KIT modified [B]	493	1.72	1.08	5.81
6	5Prime ArchivePure DNA Yeast & gram-+ KIT modified [B]	417	1.85	1.23	5.85
11	Querol *et al.* (1992) modified [C]	335.5	1.90	2.00	2.30
12	Querol *et al.* (1992) modified [C]	303.5	1.90	2.07	2.10
13	CTAB 1 [D]	719.5	2.04	1.96	4.73
15	CTAB 1 [D]	724.5	2.09	1.98	6.70
16	CTAB 2 [E]	854.5	1.90	2.04	5.99
17	CTAB 2 [E]	763.5	1.89	2.06	4.50
18	NucleoSpin Food [F]	47.5	1.19	0.99	7.16
19	NucleoSpin Food [F]	11.70	1.77	1.05	4.44
20	QIAamp DNA Stool Mini Kit [G]	2.00	2.22	0.15	0.24
21	QIAamp DNA Stool Mini Kit [G]	2.60	1.63	0.22	0.25
22	Purelink Microbiome [H]	30.13	1.35	1.58	3.06
23	GEN-IAL Simplex Easy Wine Kit [I]	220.8	/	/	2.57
24	GEN-IAL Simplex Easy Wine Kit [I]	175.1	0.80	1.28	1.90
25	GEN-IAL Simplex Easy Wine Kit [I]	670.5	1.08	2.22	7.03

[A] 5 PRIME ArchivePure DNA Yeast & gram-+ KIT; [B] modified from A adding the following steps: 3 washes of the pellet with 1 ml PBS ice cold followed by a centrifugation at 8,000 g for 5 min; add 30 µl lysozyme (40 mg/ml) to Solution 1 (cell Suspension) and invert tube 25 times; after the heating at 80 °C for 5 min, add 0.25 ml 0.5 mm sterile beads and vortex at 14,000 g for 2 min; [C] Querol *et al.* (1992) with some modifications (Vaudano *et al.*, 2016); [D] CTAB extraction (method E) with some modifications: addition of 50 µl lysozyme (30 mg/ml); incubation at 65°C for 30 min; one only step with Chloroform:Isoamyl alcohol (24:1) and before this step 100 µl of CTAB 10% were added; [E] CTAB based method from literature (Doyle and Doyle, 1987); [F] NucleoSpin Food (Macherey-Nagel, Düren, Germany); [G] QIAamp DNA Stool Mini Kit (*Qiagen,* Hilden, Germany); [H] PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific); [I] GEN-IAL Simplex Easy Wine Kit (GEN-IAL GmbH, Troisdorf, Germany)

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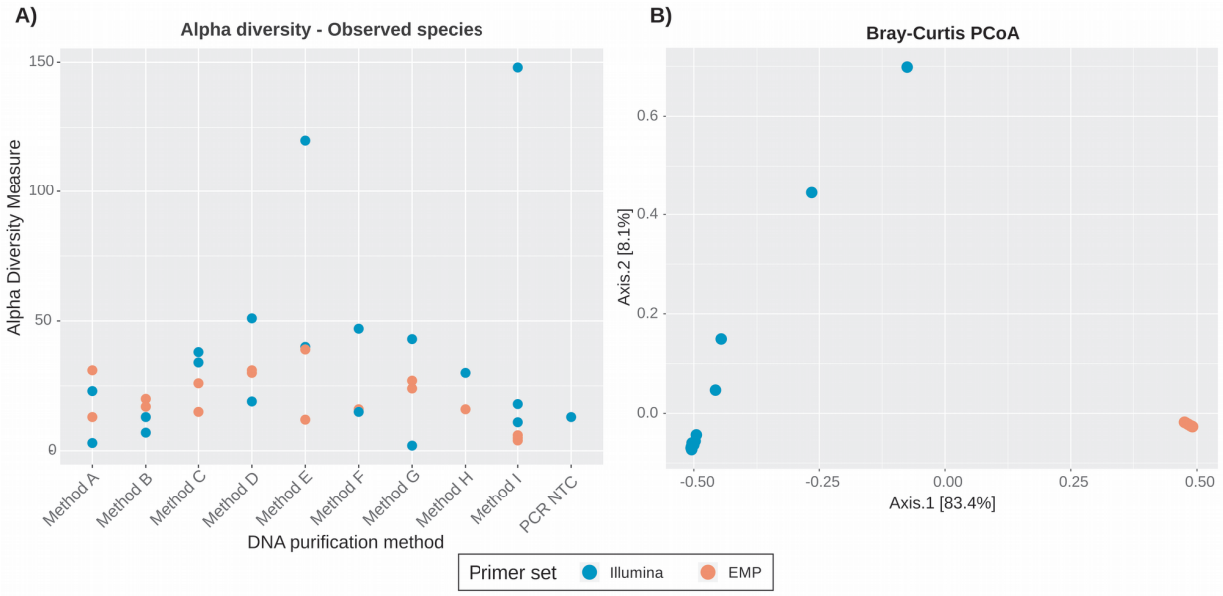


FIGURE 1. Alpha and beta diversity plots
a) Alpha diversity reported as "Observed species" for samples grouped by DNA purification method. Dot colors represent the primer set. b) Principal coordinate analysis based on Bray-Curtis dissimilarity index of the wine and NTC samples by primer set. As reported by the axis label, axis 1 shows 83.4 % of the variation in the samples, and axis 2 8.1 %, for a total of 91.5 %.

Agarose gel electrophoresis revealed that amplification was poor on DNA purified with GEN-IAL, nevertheless these samples were processed for metabarcoding.

The sequencing generated 36,031,756 reads in total (median per sample: 464,896; min: 12,621; max: 933,358). Reads for samples amplified with ILL primers were 17,911,300, while 16,446,196 for EMP primers.

Alpha diversity (Figure 1a) showed variability in number of observed species depending on the extraction method and the primer set used. ILL primers showed a higher variability within replicates compared to the EMP primers. However, no statistically significant difference was observed between purification methods or primer sets.

Beta diversity showed a clear separation of the samples based on the primer set (Figure 1b). EMP group created a tiny cluster, while ILL group showed one cluster and several outliers dispersed along both axes. Axis 1 explains 83.4% of variability in the dataset, explaining >90% of the total variability together with Axis 2 (8.1%). Finally, this difference between the two groups is statistically significant (p-value <0.001, PERMANOVA test).

The most represented genus is *Oenococcus* (89.38%), followed by *Burkholderia* (2.07 %). As graphically summarized in Figure 2, within the order *Lactobacillales*, other genera with lower abundance are *Facklamia* (0.32 %), *Lactobacillus* (0.3 7%), *Enterococcus* (0.19 %), and *Strepto-coccus* (0.11 %).

To exclude contamination, we compared the relative abundance between genera present in NTC and in the samples (Figure 2). Top genera (abundance >5 %) in NTC were *Facklamia* (26.64%), *Stenotrophomonas* (15.39 %), *Staphylococcus* (14.54 %), *Achromobacter* (7.93 %), *Propionibacterium* (7.78 %), *Methylobacterium* (6.94 %), and *Cloacibacterium* (6.49 %). These genera in the samples had the following relative abundance in decreasing order: *Stenotrophomonas* (0.76 %), *Cloacibacterium* (0.60 %), *Methylobacterium* (0.36%), *Propionibacterium* (0.17%), *Achromobacter* (0.04 %), *Staphylococcus* (0.12%), and *Facklamia* (0.01 %). The relative abundance between contaminant genera in the NTC is not conserved in the samples. Moreover, these genera are only present at a very low rate in samples.

DISCUSSION

In this study, we evaluated nine DNA purification methods starting from an inoculated

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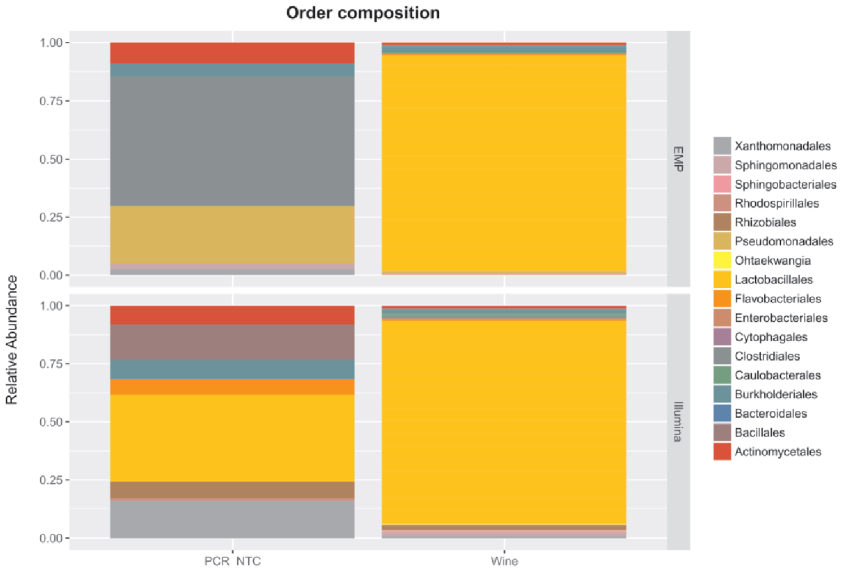


FIGURE 2. Bar plot of the order composition in NTC and in wine samples

Barbera wine after malolactic fermentation and performed a 16S rRNA gene metabarcoding with two primer pairs to evaluate the performance of the different protocols and to select the best one.

The sequencing generated good quality reads that were analyzed for the comparison within and between DNA purification methods and primer pairs. To evaluate the different 16S metabarcoding protocols from wine, we adopted the "observed species" index expecting the same output in terms of number of species, since the starting sample was the same. This parameter was considered relevant as, if one method returned fewer species, it was not able to extract DNA from the missing species. In our analysis, the "observed species" index between extraction methods did not show statistically significant differences, meaning that the methods may be comparable. To evaluate the reproducibility, since DNA was extracted in at least two replicates for each method, if the pairs returned similar values of observed species, then the method successfully extracted DNA consistently between replicates. Based on Figure 1a, we estimated that the method D combined to the EMP primers performed better than the other methods, since it returned reproducible results with a higher number of species detected. Also methods G and I were accurate, but they identified less species. For this reason, we would suggest the method D for DNA purification from wine.

The beta diversity showed a much clearer picture on the outcome of using the two primer sets. The neat separation between samples amplified with the two primer sets may be explained by their different target length, and thus to a slightly different taxonomic designation. Since the analyzed samples derive from the same wine, one should expect that all the dots representing the single replicates would be overlapped, with no variation at all. Despite this situation is hard to be realized in practice due to replicate variability, the dots representing samples amplified with EMP primers created a tiny cluster, meaning that there is no great variability within this group. On the opposite, the ILL group showed a cluster of samples and other outliers dispersed along both axes. Especially because Axis 1 explains 83.4% of the variability in the dataset, the dispersion of the samples in this direction highlights the great variability observed within samples processed using the ILL primer set, further enhanced by the dispersion along Axis 2.

Such a great difference in terms of reproducibility to describe the same microbial community may be explained by the different target size. EMP primers have a shorter target than ILL (~250 bp vs ~420 bp) and this may lead to a successful amplification of more DNA fragments, and then taxa. ILL primers, having a longer target, may fail to amplify some taxa, when the DNA integrity is compromised. Actually, the DNA integrity profile obtained with the Agilent BioAnalyzer (data not shown)

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showed a degraded DNA, with fragments between 35 and 300 nt, that is consistent with the target size. ILL primers may then amplify DNA from only some taxa and exclude others whose DNA is degraded. Suboptimal DNA quality is a frequent conditions when working with samples from the field and the ability of the primer set to catch all the diversity of a microbial community is of great importance.

The importance of metabarcoding primer selection based on the type of study to be conducted was underlined in a recent work by Poirier and colleagues. They showed that primers have an influence on the detection of *Leuconostocaceae* in metabarcoding, with ILL forward primer being more successful than the Muyzer one (Poirier *et al.*, 2018). From the results of our experiments, we suggest the use of the EMP primer set to study the wine microbiota.

Although out of the main purpose of this work, our data confirmed that, after the inoculation with a lyophilized commercial preparation of *O. oeni*, this species is the most abundant during malolactic fermentation, although other genera are present at a much lower abundance.

In our study, we adopted an inoculated wine to work with a controlled malolactic fermentation and a stabilized wine in order to better compare the outcome of the different protocols for metabarcoding. The microbiota composition of this wine may not be relevant to understand how the malolactic fermentation is regulated by a spontaneous microbiota, as usually occurs in high quality winemaking. However, this induced microbiota showed that the inoculated bacteria are not the only species contributing to the fermentation.

In conclusion, our data showed that wine microbiota may be characterized with the 16S rRNA gene metabarcoding, and paved the way to further studies aiming to describe the evolution of the microbial community in the winemaking process.

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