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DIFFERENCES BETWEEN MICROBIOTA, PHYTOCHEMICAL, ANTIOXIDANT PROFILE AND DNA FINGERPRINTING OF CABERNET SAUVIGNON GRAPE FROM SLOVAKIA AND MACEDONIA

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ABSTRACT

This study aimed to evaluate the microbiota, phytochemical, antioxidant profile and DNA fingerprinting of Cabernet Sauvignon grapes from Slovakia and R. North Macedonia. There were used two samples of grape berries (one sample from Slovakia and one from Macedonia). Each sample was analyzed in triplicate. The bacteria were cultivated on Plate count agar (PCA), microscopic filamentous fungi were cultivated on Malt extract agar (MEA). MALDI-TOF MS Biotyper mass spectrometry was used for the identification of microorganisms (bacteria and yeasts) and microscopic filamentous fungi with manuals. DPPH method was used to determine of antioxidant activity of grape berries. Phytochemical and antioxidant profiles were evaluated in grape berries samples. Total genomic DNA was extracted from mature grapes by GeneJET Plant Genomic DNA Purification Kit. The number of bacteria was higher in the sample of Macedonian grape (4.13 log CFU.g^{-1}) in comparison to the grape from Slovakia as well as the number of yeasts was also higher in the Macedonian sample (2.57 log CFU.g^{-1}). Antioxidant activity of Slovak grape berries was 0.55 mg TEAC.g^{-1} and of Macedonian grape, berries was 0.51 mg TEAC.g^{-1}. Total polyphenol content was higher in grape from Slovakia (0.81 mg GAE.g^{-1}) than in grape from Macedonia (0.77 mg GAE.g^{-1}), while total flavonoid content was 0.57 and 0.17 mg QE.g^{-1} in Slovak grape and Macedonian grape, respectively. Total phenolic acid content was higher in the sample from Macedonia (0.40 mg CAE.g^{-1}) compared to the grape from Slovakia (0.24 mg CAE.g^{-1}). Total anthocyanin content was also higher in Macedonian grape (0.46 mg.g^{-1}) compared to the Slovak sample (0.05 mg.g^{-1}). The total polymorphism for all of the used primers of 87.5% was obtained for the Macedonian sample of Cabernet Sauvignon and 89.4% for the Slovak sample.

Keywords: grape berries; bacteria; yeasts; antioxidant profile; MALDI-TOF MS Biotyper; polymorphism

INTRODUCTION

Grapes have been used for winemaking since the ancient Greek and Roman civilizations **(Ma and Zhang 2017)**. The presence of biologically active substances in fruits brings considerable benefits to consumers, whether consumed raw **(Durec et al., 2019)**. Grapes are rich in phytochemicals with many proven health benefits **(Liang et al., 2014)**. They are one of the most widely grown fruits and the total production of grapes worldwide is approximately 60 million tons **(Matthäus, 2008)**. Grapes can be categorized into grapes with edible seeds, seedless, wine grapes, table grapes, and raisin grapes **(Girard and Mazza, 1998)**. Grape seeds are rich in phenolic compounds and have potentially beneficial effects for human health such as protection against peptic ulcers, oxidative stress, tissue damage, and inflammation **(Rodríguez Montealegre et al., 2006; Kim et al., 2013)**. Grape seeds have been reported to exhibit scavenge superoxide radicals. Grape seeds are rich in flavan-3-ol, including proanthocyanidins and catechins **(El-Beshbishy, Mohamadin and Abdel-Naim, 2009)**.

Biological activities and health-promoting benefits are mostly possessed by polyphenols, which are considered to be the most important phytochemicals of grape. The phenolic compounds mainly include anthocyanins, flavanols, flavonols, stilbenes (resveratrol) and phenolic acids **(Xia et al., 2010)**.

From the vineyard to the winery, microorganisms play key roles in wine production and quality. The grapevine (*Vitis vinifera*) phyllosphere harbors diverse microbes including yeasts, filamentous fungi and bacteria that substantially modulate grapevine health, growth, and grape and wine production **(Gilbert, van der Lelie and Zarraonaindia, 2014)**.

Microbes could originate from the vineyard soil **(Morrison-Whittle and Goddard, 2018)**, air, precipitation (rainfall, hail, snow), be transported by animal vectors (bees, insects, and birds) **(Francesca et al.,**

2012; Stefanini et al., 2012; Lam and Howell, 2015), and be resident in nearby native forests **(Morrison-Whittle and Goddard, 2018)**.

Microbes that are grapevine-associated and are transferred to the must-have a profound influence on wine composition, flavor and quality **(Barata, Malfeito-Ferreira and Loureiro, 2012)**. Fermentative yeasts (primarily *Saccharomyces cerevisiae*) and lactic acid bacteria (LAB, predominantly *Oenococcus oeni*) in the must modulate the flavor and aroma of wine **(Swiegers et al., 2005)**.

In the study of **Kačániová et al. (2018)** a total of 33 species of 8 Gram-negative (20.72%), 10 Gram-positive (31.53%) bacteria and 10 yeasts species of 8 genera (47.74%) were identified with MALDI-TOF Mass Spectrometry.

Inter Primer Binding Site (iPBS) polymorphism is generated on the biological background of plant pararetroviruses, which primer binding site (PBS) is complementary to the 3′ end of the primer tRNA. In plant retrotransposons, the PBS is either complementary to the 3′ end or an internal region of the primer tRNA. The method of whole genome iPBS amplification is based on the virtually universal presence of a PBS in LTR retrotransposons **(Kalendar et al., 2010)**. This technique has been proved to be a powerful DNA fingerprinting technology without the need for prior sequence knowledge **(Fang-Yong and Ji-Hong 2014; Kalendar, Amenov and Daniyarov, 2018)**. It has the potential to discriminate among close genotypes **(Antonius-Klemola, Kalendar and Schulman, 2006)** and is highly reproducible **(Guo et al., 2014)**. Polymorphism generated by iPBS works well for both, the Gypsy and Copia LTR retrotransposons **(Melnikova et al., 2012)**.

Scientific hypothesis

Grape berries contain various microorganisms. Bacteria, yeasts and molds could be identified with MALDI TOF mass spectrometry.

There are many biologically active compounds in grape berries – flavonoids, polyphenols, phenolic acid and anthocyanins.

MATERIAL AND METHODOLOGY

Two types of grapes were studied in this work: one from Slovakia and one from Macedonia.

The phytochemical and antioxidant profile of the grape

The fresh grape berries were used for the preparation of ethanolic extract; 1 g of each sample was extracted with 20 mL of 80% ethanol for 2 h and centrifuged at 4000 g (Rotofix 32 A, Hettich, Germany) for 10 min. The supernatant was used for the measurement of antioxidant activity (DPPH) and the detection of total polyphenol, total flavonoid, and phenolic acid content.

Chemicals

All chemicals were of analytical grade and purchased from Reachem (Slovakia) and Sigma Aldrich (USA).

DPPH Method—Radical Scavenging Activity

Radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the procedures described by **Sánchéz-Moreno, Larrauri and Saura-Calixto (1998)**. An amount of 0.4 mL of extract was added to 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). The absorbance of the reaction mixture was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Radical scavenging activity of the samples was expressed as Trolox equivalent antioxidant capacity (mg TEAC.g^{-1}).

Total Polyphenol Content

The total polyphenol content of extracts was measured by the method of **Singleton and Rossi (1965)** using Folin-Ciocalteu reagent. A 0.1 mL of each sample was mixed with 0.1 mL of Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate, and 8.8 mL of distilled water. After 30 min in darkness, the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid was used as the standard and the results were expressed in mg.g^{-1} of gallic acid equivalents.

Total Flavonoid Content

Total flavonoids were determined using the modified **Willett** method **(2002)**. A 0.5 mL of sample was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 mL of 1 M potassium acetate, and 4.3 mL of distilled water. After 30 min in darkness, the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin was used as the standard and the results were expressed in mg.g^{-1} of quercetin equivalents.

Total Phenolic Acid Content

Total phenolic acid content was determined using a method of **Polish Pharmaceutical Society (2005)**. A 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnow reagent (10% $NaNO_2$ + 10% Na_2MoO_4), 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid was used as a standard and the results were expressed in mg.g^{-1} of caffeic acid equivalents.

Total Anthocyanin Content

Anthocyanin content was measured according to the method of **Fuleki and Francis (1968)** with modifications **(Lee, Durst and Wrolstad, 2005)**. For pH 1.0, a sample (0.4 mL) was diluted with 0.025 M of potassium chloride (3.6 mL). For pH 4.5, a sample was diluted (0.4 mL) with 0.4 M of sodium acetate. The absorbance of the sample was measured at 520 and 700 nm against the blank reagent (distilled water). The concentration (mg.g^{-1}) of total anthocyanins was calculated according to the following formula and expressed as cyanidin-3-glucoside (Cy-3-glc) equivalent:

A [mg.g^{-1}] = (A∗Mw∗1000)/(ε∗L), A[mg.g^{-1}] = (A∗Mw∗1000)/(ε∗L),

where: A is the absorbance difference = ($A_{520} - A_{700}$) pH 1.0 − ($A_{520} - A_{700}$), pH 4.5; M_W is the molecular weight of (Cy-3-glc) = 449.2 g.mol^{-1}; ε is the extinction coefficient of (Cy-3-glc) = 1700 cm.mol^{-1}; L the absorption; path length : 1 cm.

Microbiological analyses of grape berries samples

Five grams of berries from each grape samples were diluted in 45 mL of sterile physiological saline (0.85%). Berries were stirred on a horizontal shaker for 30 minutes. After that, the dilutions of 10^{-2} and 10^{-3} were prepared for cultivation with the spread plate method. A 0.1 mL of each dilution (10^{-2}, 10^{-3}) was placed on the surface of a solid cultivation medium. Bacteria were cultivated on Plate count agar (PCA) (Oxoid, UK), yeasts on Malt extract agar base (MEA) (Oxoid, UK) supplemented with bromocresol green (0.020 g.L^{-1}) (Centralchem®, Slovakia). Bacteria were cultivated at 37 °C for 24 – 48 h in aerobic condition, yeasts at 25 °C for five days in aerobic conditions. Growing colonies with macroscopic morphological differences were recultivated on TSA (Tryptic Soy agar, Oxoid®). Inoculated plates were cultivated at 30 °C for 48 h (TSA). After cultivation, the proteins were extracted from fresh bacterial colonies.

Sample preparation and MALDI-TOF MS measurement

One colony of each bacterial and yeast isolate was transferred into an Eppendorf vial and mixed in 300 µL of sterile water. After the addition of ethanol (900 µL), the suspension was mixed and centrifuged (13 000 g, 2 min). After removal of the supernatant, the pellets were dried at room temperature at least for 5 min. The bacterial and yeast pellets were resuspended in 20 – 50 µL of formic acid (70%) and the same amount of acetonitrile. After centrifugation (2 min at 13,000 g), a 1 µL of supernatant was spotted onto a sample position of a polished steel MALDI target plate and dried at room temperature. A 1 µL of MALDI matrix (solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile/2.5% trifluoro-acetic acid) was added to the spot and dried.

The MALDI target plate was introduced into the MALDI-TOF mass spectrometer (Bruker, Germany) for automated measurement and data interpretation. MALDI-TOF profile mass spectra were imported into the MALDI Biotyper 3.0 software and processed automatically after measurement. The logarithm of the score (log[score]) was displayed as the matching result. The MALDI Biotyper output was a log(score) between 0 and 3.0, which was calculated from a comparison of the peak list from an unknown isolate with the reference MSP in the database. A log(score) ≥1.7 indicated identification at the genus level, log(score) ≥2.0 was set as the threshold for a match at the species level. Isolates with ≥2.0 were accepted as a correct identification.

DNA extraction and iPBS profiles amplification

Total genomic DNA was extracted from mature grapes by GeneJET Plant Genomic DNA Purification Kit (Thermo Fisher) following the instructions of the manufacturer. The iPBS primers 1845, 1846 and 1886 were used for the fingerprints amplification **(Kalendar et al., 2010)**. The following iPBS PCR profile was used for the Combi PPP 2x MasterMix (Top Bio) and 50 ng of DNA: 94 °C – 5 min; 35 cycles of : 95 °C 1 minute; 55 °C 2 minutes; 72 °C 3 minutes with final 72 °C 10 minutes. Amplified fragments were analyzed in 6% PAGE and scored for the presence or absence of amplicons in GelAnalyser software. UPGMA analysis and dendrogram construction was performed in SYNTAX software using a Jaccard coefficient of similarity to define relationships between individual obtained iPBS profiles for analyzed samples of Cabernet Sauvignon.

Statistical analysis

All experiments were carried out in triplicate and standard deviations for replication as well as T-tests were calculated using MS Excel.

RESULTS AND DISCUSSION

The phytochemical and antioxidant profile of studied grapes (or grape samples)

According to many authors, the antioxidant activity of grape berries and wines results mainly from their phenolics, whereas the phenolic content and composition depend on the grape variety, vineyard location, cultivation system, climate, soil types, vine cultivation practices, harvesting time, production process and aging **(Shahidi and Naczk, 1995).**

DPPH·method is one of the most popular methods for detecting the antioxidant activity of wine **(Wang, 2008)**. The experimental results indicate that the higher the amount of antioxidants, the lower is the concentration of remaining DPPH and the stronger is the radical-scavenging activity **(Jiang and Sun, 2018)**.

The antioxidant activity of Slovak grape berries was 0.55 mg TEAC.g^{-1} and antioxidant activity of Macedonian grape were 0.51 mg TEAC.g^{-1}. **Jiang and Zhang (2012)** reported that the contents of phenolic compounds and the levels of antioxidant activity in the wine samples greatly varied with cultivar and environmental factors of wine growth.

The value of total polyphenols was 0.81 mg GAE.g^{-1} in grape from Slovakia and 0.77 mg GAE.g^{-1} in grape berries from Macedonia. Total flavonoids were 0.57 mg and 0.17 QE.g^{-1} in Slovak and Macedonian grape berries, respectively. Phenolic compounds, which are abundant in grape berries and wines, play one of the most important roles in the quality of grapes and wines. They strongly contribute to the color, mouthfeel and palatability of red wines **(Lesschaeve and Noble, 2005)**. Moreover, polyphenols also exert many favorable effects on human health, such as the inhibition of atherosclerosis, coronary heart disease, and various cancer types **(Yilmaz and Toledo, 2004)**. Total phenolic acid content was 0.24 and 0.40 mg CAE.g^{-1} in grape from Slovakia and grape from Macedonia, respectively.

Table 1 Antioxidant activity, total polyphenol, flavonoid, phenolic acid and anthocyanin content of analyzed grape.

Samples	DPPH mg TEAC.g^{-1}	TPC mg GAE.g^{-1}	TFC mg QE.g^{-1}	TPAC mg CAE.g^{-1}	TAC mg.g^{-1}
Slovak Cabernet Sauvignon grape	0.55 ±0.01	0.81 ±0.05	0.57 ±0.05	0.24 ±0.07	0.05 ±0.01[a]
Macedonia Cabernet Sauvignon grape	0.51 ±0.15	0.77 ±0.09	0.17 ±0.02	0.40 ±0.01	0.46 ±0.03[a]

Note: *DPPH* - 2,2-difenyl-1-picrylhydrazyl *TPC* total polyphenol content, *TFC* total flavonoid content, *TPAC* total phenolic acid content, *TAC* total anthocyanin content, *GAE* gallic acid equivalent, *QE* quercetin equivalent, *CAE* caffeic acid equivalent, *FM* fresh matter; [a] significant difference of analysed parameter.

Table 2 Microorganisms counts isolated from wine grapes in log CFU.g^{-1}.

Sample	Bacteria	Yeasts
Slovak Cabernet Sauvignon grape	3.57 ±0.29	2.34 ±0.27
Macedonian Cabernet Sauvignon grape	4.13 ±0.08	2.57 ±0.18

Table 3 Microorganisms isolated from wine grape berries.

Slovak Cabernet Sauvignon grape	*Alternaria* sp., *Bacillus endophyticus, Escherichia coli, Hanseniaspora uvarum, Issatchenkia orientalis, Lactobacillus fermentum, Leuconostoc mesenteroides* susp. *mesenteroides, Metschnikowia pulcherrima, Pantoea agglomerans, Stenotrophomonas maltophilia, Yarrowia lipolytica, Botrytis cinerea, Cladosporium* sp., *Ignatzschineria indica, Kazachstania exigua, Kluyveromyces marxianus, Lactobacillus paracasei, Penicillium expansum*
Macedonian Cabernet Sauvignon grape	*Alternaria* sp., *Bacillus endophyticus, Escherichia coli, Hanseniaspora uvarum, Issatchenkia orientalis, Kazachstania exigua, Lactobacillus fermentum, Leuconostoc mesenteroides* susp. *mesenteroides, Metschnikowia pulcherrima, Pantoea agglomerans, Stenotrophomonas maltophilia, Yarrowia lipolytica, Bacillus cereus, Issatchenkia orientalis, Lactobacillus paracasei*

Macedonian Cabernet-Savignon			Slovak Cabernet-Savignon		
1845 iPBS profile	1846 iPBS profile	1886 iPBS profile	1845 iPBS profile	1846 iPBS profile	1886 iPBS profile
1	1	0	1	0	0
0	0	1	0	0	1
0	0	1	1	0	0
1	0	0	0	0	0
0	1	0	1	1	0
1	1	0	0	0	0
1	0	0	0	0	1
0	0	0	1	0	0
0	0	0	1	0	0
1	1	1	1	1	0
1	0	0	1	0	1
0	0	1	1	0	1
1	1	0	1	1	0
0	0	0	0	0	1
1	0	0	1	0	0
1	1	1	1	1	1
0	0	0	1	0	0
0	1	1	1	1	0
0	1	1	1	0	1
0	0	0	1	0	0
1	1	1	1	1	1

Figure 1 Amplification profiles of analysed samples of Cabernet Sauvignon.

Yang, Martinson and Liu (2009) analyzed the total phenolic contents of 14 wine grapes. Among all the grape varieties analyzed, Cabernet Franc and Pinot Noir had the highest total phenolic content (424.6 ±3.8 and 396.8 ±12.4 mg of gallic acid equivalents per 100 g of grape, respectively), followed by Concord, Sheridan, Chancellor, Marechal Foch, Catawba, DeChaunac, Riesling, Niagara, Vidal Blanc, Baco Noir, Cayuga White, and Chardonnay.

Mitić et al. (2012) measured the total flavonoid content of the 7 grape extracts, 'Cabernet Sauvignon' presented the highest flavonoid content (146.2 mg of CE.100 g^{-1} of f.w.), followed by 'Merlot', 'Vranac', 'Muscat Hamburg', 'Prokupac', 'Ribier', and 'Cardinal'. **Ivanova, Stefova and Chinnici (2010)** measured lower values 60.3 CE.100g^{-1} f.w. the average total flavonoids content of grape cultivars 'Vranec', 'Cabernet Sauvignon', and 'Muscat Hamburg'.

Anthocyanins are natural pigments, responsible for a wide range of colors in grapes and red wines. The anthocyanins in red grapes vary greatly with the species, maturity, production area, seasonal conditions, and yield of the fruit **(Mitić et al., 2012)**. The total anthocyanin content was 0.05 mg.g^{-1} in grape from Slovakia and 0.46 mg.g^{-1} in grape from Macedonia. Table 1 compares data related to antioxidant activity, total polyphenol, flavonoid, phenolic acid and anthocyanin content of analyzed grape. The statistical difference was found only in TAC.

Microbiota of grape

The surface of grape berries represents a comprehensive natural reservoir of bacterial microbiota originating from the surrounding environment **(Zarraonaindia et al., 2015)**. The value of bacteria was 3.57 log CFU.g^{-1} in Slovak grape and 4.13 log CFU.g^{-1} in Macedonian grape berries. The value of yeasts was 2.34 log CFU.g^{-1} in Slovak grape and 2.57 log CFU.g^{-1} in Macedonian grape (Table 2). Numerous yeast genera and species are found during the production of wine. The low pH of the wine, high sugar content, rapidly generated anaerobic conditions, and presence of phenolic compounds create the ideal environment to support the growth of yeasts and to enrich these organisms with other microbes **(Fleet, 2003)**.

Grapes have a complex microbial ecology including filamentous fungi, yeasts, and bacteria with different physiological characteristics and effects upon wine production. Some species are only found in grapes, such as parasitic fungi and environmental bacteria, while others can survive and grow in wines, constituting the wine microbial consortium. This consortium covers yeast species, lactic acid bacteria, and acetic acid bacteria **(Barata, Malfeito-Ferreira and Loureiro, 2012).**

Bacterial populations are usually several orders of magnitude lower than those of yeasts in sound grapes. Lactic acid bacteria have counts lower than 10^2 CFU.g^{-1} **(Francesca et al., 2011)**.

Table 3 presents microorganisms isolated from Slovak and Macedonian grape berries. Worldwide surveys indicate that sound grapes are colonized by a wide variety of yeast species without any obvious explanation. However, the variety may be reduced to relatively few groups of similar physiological characteristics. For instance, the ubiquitous *Candida* spp. and *Pichia* spp. are highly heterogeneous, and new species are likely to be found in each new survey due to the accuracy of molecular identifications is constantly increasing **(Rao et al., 2007)**.

Grapevine bacteria play a key role not only in plant health but also in crop quality and yields, which can influence the winemaking process **(Nisiotou et al., 2011)**. Numerous studies have analyzed the presence of yeast on the surface of grapes and many have indicated that *Saccharomyces cerevisiae* is only present in small numbers on healthy grapes **(Pretorius, 2000)**. *Saccharomyces* can be found in grape musts, but the populations are often less than 50 CFU.mL^{-1} **(König, Unden and Fröhlich, 2009)**. We did not isolate *Saccharomyces cerevisiae* in our study.

The yeast populations of grapes generally comprise between 10^2 and 10^4 cells.g^{-1} **(Fleet et al., 2002)**, but higher values have also been reported. *Hanseniaspora uvarum* appears to be the most common grape berry species worldwide, which is consistent with its predominance at the beginning of spontaneous must fermentations. Like yeasts, lactic acid bacteria are also found in vineyards **(Lonvaud-Funel, 1999)**.

The microbiota of grapes also includes fungi that may dominate under favorable weather conditions accompanied by inefficient phytochemical utilization. Fungal obligate parasites can penetrate through the intact grape skin by their own biochemical and mechanical activities and are responsible for high economic losses. The main species are the oomycete *Plasmopara viticola,* responsible for downy mildew, and the ascomycetes *Erysiphe necator* (powdery mildew), *Elsinoë ampelina* (anthracnose), *Guignardia bidwellii* (black rot) and *Pseudopezicula tracheiphila* (rotbrenner) **(Barata, Malfeito-Ferreira and Loureiro, 2012)**.

DNA fingerprinting

The variability in polymorphism length was inspected among the Macedonian and Slovak Cabernet Sauvignon grapes using an iPBS markers 1845, 1846 and 1886. The total number of obtained iPBS fragments was 57 which were distributed to 21 levels. The average number of fragments per primer was 9.5. Their size ranged from 378 bp up to the 882 bp. The level of the shortest fragments was present in both of the analyzed varieties for all of the used primers (Figure 1). The highest number of obtained fragments per one primer was 16 fragments for Cabernet Sauvignon from Slovak growing conditions analyzed by primer 1845. The lowest number – 6 fragments for Cabernet Sauvignon from Slovak growing conditions analyzed by primer 1846. One unique fragment was amplified for the Cabernet-Sauvignon from Macedonian growing conditions analyzed by primer 1845 with the length 787 bp.

The total polymorphism for all of the used primers of 87.5% was obtained for the Macedonian sample of Cabernet Sauvignon and 89.4% for the Slovak sample. The most similar iPBS profiles of Slovak and Macedonian samples of Cabernet Sauvignon grapes were obtained for the primer 1846 (Figure 2).

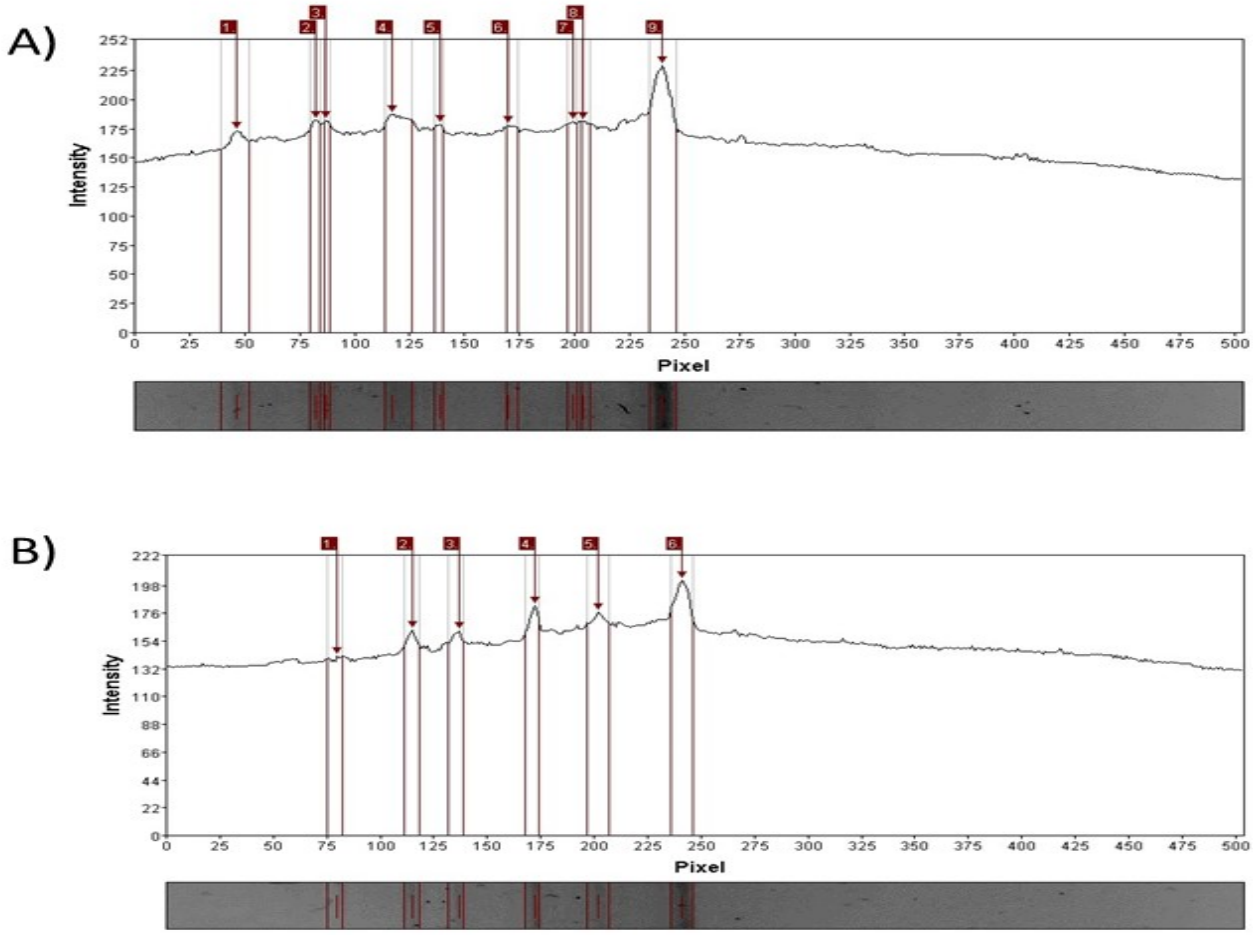

Figure 2 Analysis of length of obtained 1846 fragments for samples from Moldavia(A) and Slovakia (B) evaluated by software GelAnalyzer.

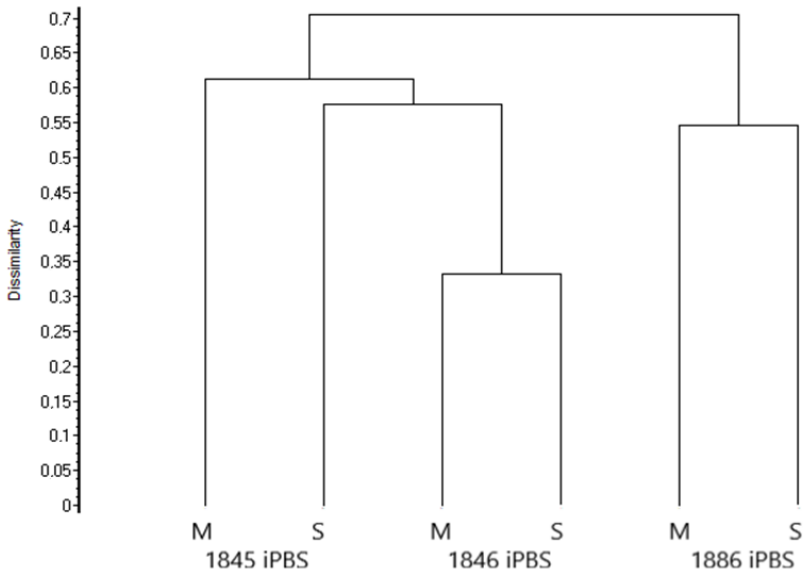

Figure 3 Dendrogram of obtained iPBS profiles of analysed samples of Cabernet-Sauvignon.

The analysis of the relationships of obtained iPBS amplicon profiles was performed by the clustering method using the UPGMA analysis (Figure 3). A clear effect of primer can be seen preferentially to the provenience of the analyzed samples. Profiles generated by primers 1845 and 1846 were grouped and the profiles of 1886 primer were separated as a new branch at the level of 0.7. Here again, the highest similarity of 1846 iPBS profiles of Macedonian and Slovak samples was confirmed.

The PBS primed PCR generated markers are reported to be very effective for extensive intraspecific polymorphism detecting, including in the study of clonal variability. Genotyping by iPBS markers was used for finding differences between varieties and their clones as well as one of the tools for grapevine collection management **(Butorac et al., 2018; Drori et al., 2017)**. Shannon index with the value of 0.137 was reported for Cabernet Sauvignon for six genotypes by **Milovanov et al. (2019)** when a total of 30 PBS primers were used.

CONCLUSION

In our study, the Slovak grape berries sample contained a higher concentration of polyphenols and flavonoids, but a lower concentration of phenolic acids and anthocyanins in comparison to the Macedonian grape. The number of yeasts and bacteria was higher in grape berries from Macedonia. Weather and cultivation conditions can affect the content of biologically active components as well as microorganisms in grape berries.

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