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Potravinarstvo Slovak Journal of Food Sciences
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Slovak Journal of Food Sciences

Potravinarstvo Slovak Journal of Food Sciences
vol. 13, 2019, no. 1, p. 174-181
doi: https://doi.org/10.5219/1047
Received: 9 February 2019. Accepted: 28 March 2019.
Available online: 25 March 2019 at www.potravinarstvo.com

ISSN 1337-0960 (online)

MICROBIOTA OF DIFFERENT WINE GRAPE BERRIES

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ABSTRACT

The wine grape berries share a complex microbial ecology including filamentous fungi, yeasts and bacteria. The microbiota reveals different physiological characteristics and depends on the grape ripening stage and the availability of nutrients with different effect on wine production. The microbiota of grape berries (n = 12) was isolated and identified in the present study. The samples were collected in September 2018. Grape berries were obtained from Vrbovo vineyard located in Slovakia. The grape berries investigated belonged to Blue Frankish, Cabernet Sauvignon, Chardonnay, Dornfelder, Feteasca regala, Green Veltliner, Irsai Oliver, Müller Thurgau, Pálava, Pinot Blanc, Rhinriesling and Welschriesling varieties. The microorganisms were cultivated on Malt extract agar (MEA) at 25 °C for five days in aerobically for microscopic filamentous fungi and Tryptone Soya agar (TSA) at 37 °C for 24 – 48 h aerobically for bacteria and yeasts. Total bacterial counts on different wine grape berries ranged from 2.57 ±0.09 in Chardonnay to 4.39 ±0.21 log CFU.g^{-1} in Pálava. Microscopic filamentous fungi count ranged from 1.18 ±0.03 in Blue Frankish to 2.60 ±0.17 log CFU.g^{-1} in Welschriesling. MALDI-TOF MS Biotyper mass spectrometry was used for identification of microorganisms (bacteria and yeasts) and microscopic filamentous fungi with manuals. The most identified microscopic fungal species was *Alternaria* sp., for yeasts *Issatchenkia orientalis* and *Leuconostoc mesenteroides* subsp. *mesenteroides* for bacteria.

Keywords: microbiota; grape berries; identification; MALDI-TOF MS Biotyper

INTRODUCTION

The *V. vinifera* phyllosphere is colonized by bacteria and fungi which modulate health, development and quality of grape and the produced wine characteristics **(Barata, Malfeito-Ferreira andLoureiro, 2012)**. The grape surface inhabiting microorganisms are sensitive to environmental changes during wine fermentation and cannot survive in low pH, ethanolic and anaerobic conditions. At the same their metabolic activity on the grape surface can have consequences for wine quality, e.g. metabolic changes produced by phytopathogenic fungi **(Hong et al., 2011)**.

The grape microbiota more often demonstrates the beneficial effect, and the participation of microbiota in wine fermentations can improve the sensory characteristics of wines **(Ciani et al., 2010)**. Studies of microbiota involved in wine fermentations allowed a discovery of microbial species, which show positive enological properties. The application of those microorganisms with *Saccharomyces* yeasts was commercialized in winemaking **(Ciani et al., 2010)**. With improvement of detection and identification methods in winemaking, a growing number of microorganisms were recognized as active contributors in wine fermentations with significant improvement of sensory qualities of wine **(Ciani et al., 2010)**.

Regional wines characteristics potentially are influenced by microbial biogeography, which is another important factor for winemaking. Traditional winemaking relies mostly on native grape microbiota for fermentations. This practice thought to enhance the regional typicity. The role of microorganisms is well described for grape health, fruit and wine quality **(Barata, Malfeito-Ferreira and Loureiro, 2012)**, but the effect of grape microbiota on regional characteristics of wines is still of limited knowledge. The effect of geographic region, grape variety, and climatic factors influence the bacterial and fungal communities of wine grapes was shown through the growing years **(Bokulich et al., 2014)**. The regional fungal biodiversity of grapes was demonstrated also globally **(Taylor et al., 2014; Gayevskiy and Goddard, 2012; Pinto et al., 2015)**. The local *Saccharomyces cerevisiae* strains, the principal yeast species for wine fermentations purposes, resulting in distinct wine chemical compositions, thus the role of regional microbiota is important for winemaking **(Knight et al., 2015).**

Study of microbiota of grape berries was conducted in Slovakia **(Kačániová et al., 2018).** A total of 33 species of 8 Gram-negative (G$^-$, 20.72%) and 10 Gram-positive (G$^+$, 31.53%) bacteria and 10 yeasts species of 8 genera (47.74%) were identified with MALDI-TOF Mass Spectrometry. These results show that the yeasts were the most common group pf microorganisms isolated from grapes, but the yeasts and bacteria were isolated from each

grape variety. Bacteria counts were higher than yeast. The highest counts of yeast species were identified in Irsai Oliver (10.06%), Pálava, Pinot Blanc and Rheinriesling (9.43%) grape varieties **(Kačániová et al., 2018).**

Scientific hypothesis

Grape berries contain bacteria, yeasts and moulds, which could be identified with MALDI-TOF mass spectrometry. Microbial ecology of grapes could affect the wine grape berries health. Accurate identification of wine grape berries microbiota is essential to understand the grape microbial ecology.

MATERIAL AND METHODOLOGY

Wine grape berry samples

Twelve grape samples from wineyard in Vrbové located in the Small Carpathian wine region were used in this experiment. Ripe grape bunches were collected into sterile polyethylene bags and transported to the laboratory for microbiological analyses. The grape samples of following varieties were investigated: Blue Frankish, Cabernet Sauvignon, Chardonnay, Dornfelder, Feteasca regala, Green Veltliner, Irsai Oliver, Mūller Thurgau, Pálava, Pinot Blanc, Rhinriesling and Welschriesling.

Microbiological analyses of grape berries samples

Five grams of grape berries from each variety were diluted with 45 mL of sterile physiological saline (0.85%). Berries were stirred on a horizontal shaker for 30 min. After that, the dilutions of 10^{-2} and 10^{-3} were prepared for cultivation of sampled with spread plate method. A 0.1 mL of each dilution (10^{-2}, 10^{-3}) was cultivated on Plate count agar (PCA) (Oxoid, UK) and on Malt extract agar base (MEA) (Oxoid, UK) supplemented with bromocresol green (0.020 g.L^{-1}) (Centralchem®, Slovakia). Inoculated PCA agars were cultivated at 37 °C for 24 – 48 h aerobically. Microscopic filamentous fungi were cultivated at 25 °C for five days aerobically. The identification of fungal species was done according to the manuals of **Samson et al. (2002)**, **Samson and Frisvad (2004)**, **Pitt and Hocking (2009)**.

Growing colonies with macroscopic morphological differences were recultivated on TSA (Tryptic Soya agar, Oxoid®) and inoculated plates were cultivated at 30 °C or 25 °C for 24 h for bacteria and yeasts, respectively. After cultivation, the proteins were extraction was done.

Sample preparation and MALDI-TOF MS measurement

One colony of each bacterial and yeast isolate was transferred into an Eppendorf vial and mixed with 300 µL of sterile water. After addition of ethanol (900 µL), the suspension was mixed and centrifuged (13 000 g, 2 min). After removal of 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 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 **(Kačániová et al., 2018)**.

Climatic conditions during the wine grape harvest

Climatic conditions during the harvest were characterized by air temperature (Figure 1), soil temperature (Figure 2), and cumulative rainfall (Figure 3).

Statistic analysis

All experiments were carried out in triplicate and standard deviations for replication were calculated. The experimental data were subjected to analysis of variance (Duncan's test) at a 95% confidence level (software XL STAT, 2019).

RESULTS AND DISCUSSION

In our study, the total bacteria counts isolated from different wine grape berries ranged from 2.57 ±0.09 in Chardonnay to 4.39 ±0.21 log cfu.g^{-1} in Pálava (Table 1). Microscopic filamentous fungi count ranged from 1.18 ±0.03 in Blue Frankish to 2.60 ±0.17 log cfu.g^{-1} in Welschriesling. **Kántor et al. (2015)** found the bacteria counts on Acetobacter agar (AA) from 1.76 to 2.80 log cfu.mL^{-1}. The highest counts of acetic acid bacteria on AA agar was found in grape variety Blaufränkisch (2.80 log cfu.mL^{-1}). Lactic acid bacteria (LAB) counts on MRS agar ranged from 0.48 to 2.06 log cfu.mL^{-1}, but the LAB was not isolated from white grape varieties.

Table 1 Microorganisms counts isolated from wine grape berry varieties in log cfu.g^{-1}.

Grape type	TSA	MEA
Blue Frankish	4.42 ±0.16^{a}	1.18 ±0.03^{c}
Cabernet Savignon	4.42 ±0.09^{a}	2.51 ±0.09^{ab}
Chardonnay	2.57 ±0.09^{d}	2.35 ±0.16^{b}
Dornfelder	3.77 ±0.12^{b}	1.25 ±0.06^{c}
Feteasca regala	3.75 ±0.07^{b}	2.44 ±0.11^{ab}
Green Veltliner	3.43 ±0.20^{c}	2.35 ±0.17^{b}
Irsai Oliver	3.85 ±0.09^{b}	2.37 ±0.14^{ab}
Mūller Thurgau	3.55 ±0.07^{bc}	2.49 ±0.06^{ab}
Pálava	4.39 ±0.21^{a}	1.18 ±0.04^{c}
Pinot Blanc	3.64 ±0.13^{bc}	2.44 ±0.15^{ab}
Rhinriesling	3.78 ±0.19^{b}	2.43 ±0.08^{ab}
Welschriesling	3.41 ±0.16^{c}	2.60 ±0.17^{a}

Note: TSA-Tryptic Soya agar , MEA-Malt extract agar; mean ± standard deviation; different letters in column mean that values were significantly different.

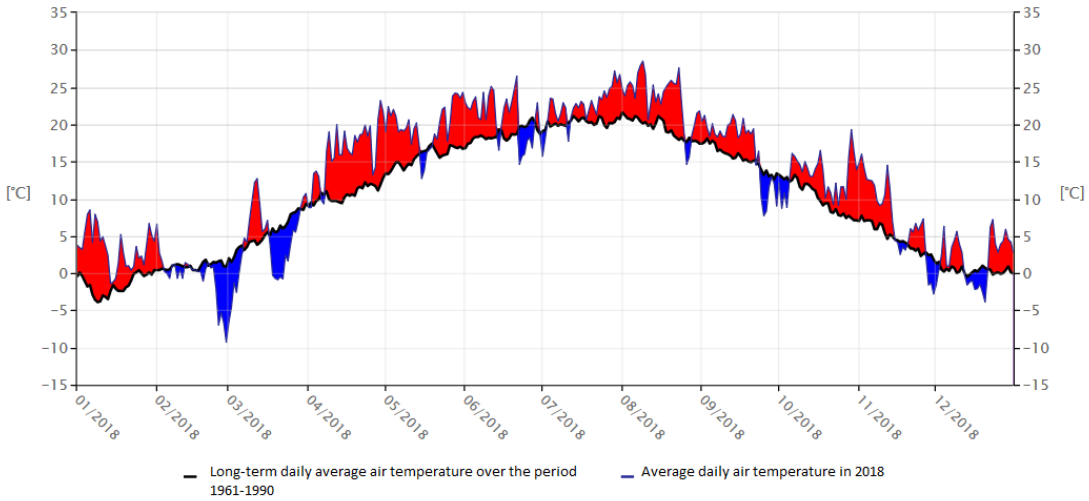

Figure 1 Average daily air temperature in Vrbové in 2018 (www.shmu.sk).

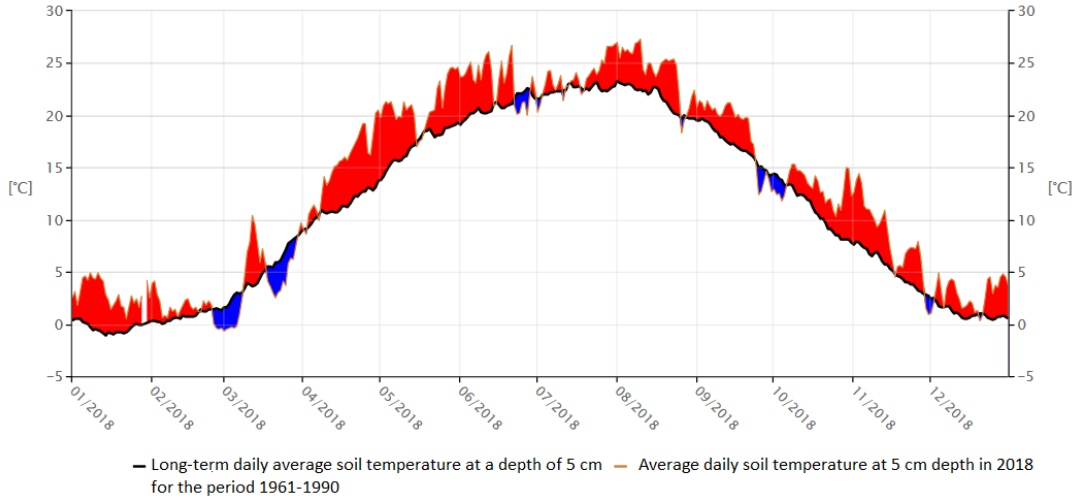

Figure 2 Average daily soil temperature in Vrbové in 2018 (www.shmu.sk).

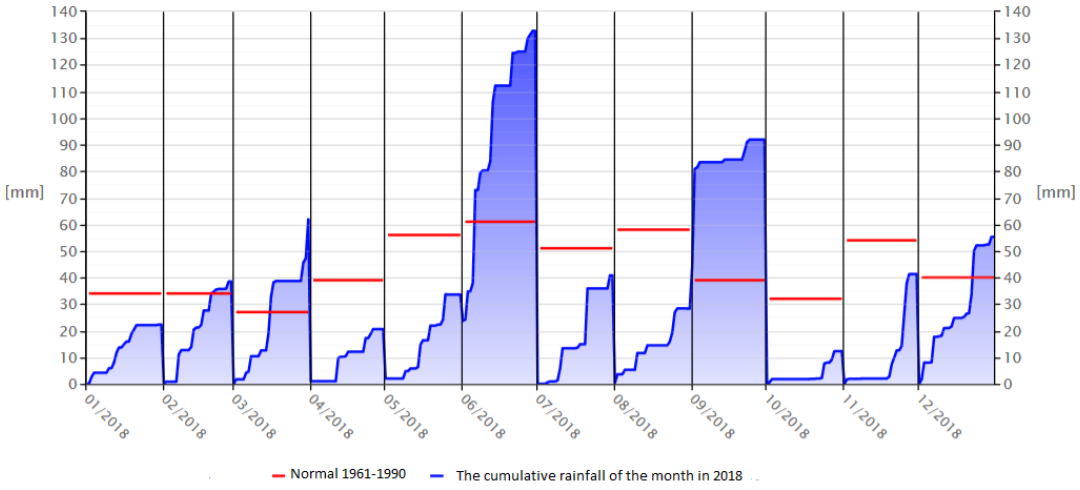

Figure 3 The cumulative rainfall in Vrbové in 2018 (www.shmu.sk).

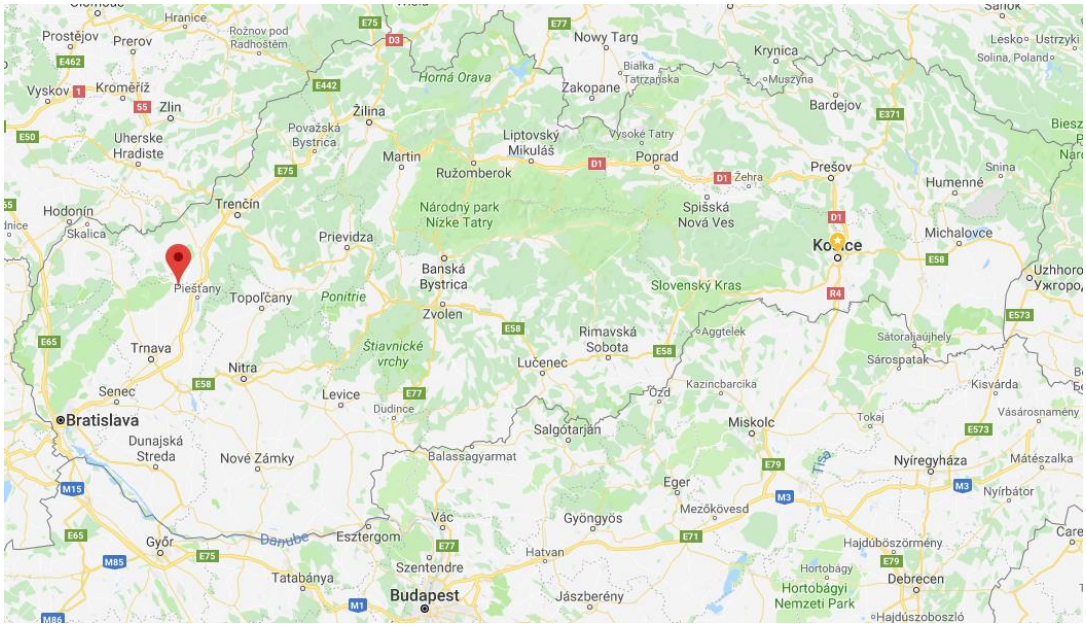

Figure 4 Vrbové, Slovakia – location.

Table 2 Microorganisms isolated from different wine grape berries varieties

Grape variety	Isolated microorganisms
Blue Frankish	*Alternaria* sp., *Arthrobacter koreensis*, *Bacillus cereus*, *Candida magnoliae*, *Escherichia coli*, *Hanseniaspora uvarum*, *Issatchenkia orientalis*, *Kazachstania exigua*, *Kluyveromyces marxianus*, *Lactobacillus acidophilus*, *Lactobacillus paracasei*, *Leuconostoc mesenteroides* susp. *mesenteroides*, *Pantoea agglomerans*, *Rhodotorula glutinis*, *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia*, *Yarrowia lipolytica*
Cabernet Savignon	*Alternaria* sp., *Arthrobacter koreensis*, *Bacillus cereus*, *Botrytis cinerea*, *Cladosporium* sp., *Enterobacter cloacae*, *Hanseniaspora uvarum*, *Ignatzschineria indica*, *Issatchenkia orientalis*, *Kazachstania exigua*, *Lactobacillus acidophilus*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Metschnikowia pulcherrima*, *Micrococcus luteus*, *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia*, *Yarrowia lipolytica*
Chardonnay	*Alternaria* sp., *Bacillus endophyticus*, *Escherichia coli*, *Hanseniaspora uvarum*, *Issatchenkia orientalis*, *Kazachstania exigua*, *Lactobacillus fermentum*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Metschnikowia pulcherrima*, *Pantoea agglomerans*, *Stenotrophomonas maltophilia*, *Yarrowia lipolytica*
Dornfelder	*Alternaria* sp., *Arthrobacter koreensis*, *Bacillus cereus*, *Hanseniaspora uvarum*, *Ignatzschineria indica*, *Issatchenkia orientalis*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, *Leuconostoc mesenteroides* susp. *mesenteroides*, *Pantoea agglomerans*, *Rhodotorula glutinis*, *Yarrowia lipolytica*

Table 2 Microorganisms isolated from different wine grape berries varieties (continue)

Grape variety	Isolated microorganisms
Feteasca regala	*Bacillus endophyticus, Candida magnoliae, Escherichia coli, Hanseniaspora uvarum, Kazachstania exigua, Lactobacillus fermentum, Lactobacillus paracasei, Leuconostoc mesenteroides* subsp. *mesenteroides, Micrococcus luteus, Penicillium expansum, Staphylococcus epidermidis*
Green Veltliner	*Alternaria* sp., *Bacillus cereus, Hanseniaspora uvarum, Issatchenkia orientalis, Lactobacillus fermentum, Lactobacillus paracasei, Leuconostoc mesenteroides* subsp. *mesenteroides, Metschnikowia pulcherrima, Pantoea agglomerans, Stenotrophomonas maltophilia, Yarrowia lipolytica*
Irsai Oliver	*Bacillus endophyticus, Cladosporium* sp., *Hanseniaspora uvarum, Issatchenkia orientalis, Kazachstania exigua, Kluyveromyces marxianus, Lactobacillus fermentum, Lactobacillus paracasei, Leuconostoc mesenteroides* subsp. *mesenteroides, Metschnikowia pulcherrima, Penicillium expansum, Rhodotorula glutinis, Staphylococcus epidermidis*
Müller Thurgau	*Bacillus cereus, Cladosporium* sp., *Hanseniaspora uvarum, Ignatzschineria indica, Issatchenkia orientalis, Kazachstania exigua, Kluyveromyces marxianus, Lactobacillus paracasei, Leuconostoc mesenteroides* subsp. *mesenteroides, Metschnikowia pulcherrima, Micrococcus luteus, Penicillium expansum, Stenotrophomonas maltophilia*
Pálava	*Aeromonas hydrophila, Alternaria* sp., *Aspergillus niger, Cladosporium* sp., *Hanseniaspora uvarum, Issatchenkia orientalis, Lactobacillus fermentum, Lactobacillus paracasei, Metschnikowia pulcherrima, Pantoea agglomerans, Staphylococcus epidermidis, Stenotrophomonas maltophilia, Yarrowia lipolytica,*
Pinot Blanc	*Bacillus endophyticus, Botrytis cinerea, Cladosporium* sp., *Hanseniaspora uvarum, Ignatzschineria indica, Kazachstania exigua, Kluyveromyces marxianus, Lactobacillus paracasei, Leuconostoc mesenteroides* susp. *mesenteroides, Metschnikowia pulcherrima, Penicillium expansum, Stenotrophomonas maltophilia*
Rhinriesling	*Alternaria* sp., *Bacillus endophyticus, Hanseniaspora uvarum, Issatchenkia orientalis, Lactobacillus fermentum, Lactobacillus paracasei, Metschnikowia pulcherrima, Pantoea agglomerans, Penicillium expansum, Rhodotorula glutinis, Staphylococcus epidermidis, Stenotrophomonas maltophilia*
Welschriesling	*Alternaria* sp., *Bacillus licheniformis, Candida magnoliae, Hanseniaspora uvarum, Issatchenkia orientalis, Kazachstania exigua, Lactobacillus fermentum, Lactobacillus paracasei, Leuconostoc mesenteroides* susp. *mesenteroides, Metschnikowia pulcherrima, Staphylococcus epidermidis, Stenotrophomonas maltophilia*

The highest LAB counts were found in grape variety Cabernet sauvignon of 2.06 log cfu.mL^{-1}, and the highest counts of LAB were detected in Blaufränkisch grape variety. The yeasts count on Sabouraud dextrose agar (SDA) ranged from 2.47 log cfu.mL^{-1} to 2.76 log cfu.mL^{-1}, and the highest counts were on Blaufränkisch grape variety surface. In generally, limited yeast diversity and low bacteria counts (10 – 10^3 CFU.mL^{-1}) were detected on immature grape berries, but the yeasts count increased to 10^4 – 10^6 CFU.mL^{-1} as the grapes were ripe enough to harvest. During ripening, the sugars diffuse from the inner tissues of the grape to the surface, that facilitating yeast

growth. Unripe grapes mostly harbour *Rhodotorula*, *Cryptococcus* and *Candida* species. These species could be isolated from mature, ripe grapes, however, the apiculate yeasts as *Hanseniaspora* (anamorph *Kloeckera*) and *Metschnikowia*, were mostly distributed. *Hanseniaspora* (*Kloeckera*), *Candida* and *Metschnikowia* species, as well as species of *Saccharomyces* and *Zygosaccharomyces* has increased incidence on the damaged grapes **(Fleet, 2003)**.

In our samples, different bacterial, yeasts and fungal species were found (Table 2). The most abundant microscopic filemanous fungi were: *Alternaria* sp., *Aspergillus niger*, *Botrytis cnerea*, *Cladosporium* sp. and *Penicillium expansum*. *Alternaria* sp. was found in 8 (66.7%) wine grape berries samples. Altogether nine yeast species were isolated: *Candida magnoliae*, *Hanseniospora uvarum*, *Ignatzschineria indica*, *Issatchenkia orientalis*, *Kazachstania exigua*, *Kluyveromyces marxianus*, *Metschnikowia pulcherrima*, *Rhodotorula glutinis* and *Yarrowia lipolityca*. *Issatchenkia orientalis* was the most abundant yeast, which was found in 10 grapes varieties (83.33%). In total, 14 bacterial species were isolated: *Arthrobacter koreeniss*, *Bacillus endophyticus*, *B. cereus*, *B. licheniformis*, *Escherichia coli*, *Enterobacter cloacae*, *Lactobacillus acidophilus*, *L. paracasei*, *L. feremntum*, *Leuconostoc mesenteroides* subs. *mesenteroides*, *Micrococcus luteus*, *Pantotea agglomerans*, *Staphylococcus epidermidis* and *Stenotrophomonas maltophilia*. The most distributed bacterial species was *Leuconostoc mesenteroides* subs. *mesenteroides*, which was isolated from 10 grape berry varieties (83.33%).

The microorganisms can contaminate from different environmental sources. The origin of microorganisms could be the vineyard, can be residents of the winery flora, or can be transmitted with insects such as fruit flies and, bees **(Fleet et al., 2002)**. Over twenty yeast species have been identified in wines **(Renouf et al., 2007)**. *Hanseniaspora uvarum* (anamorph: *Kloeckera apiculata*), *Metschnikowia pulcherrima* (anamorph: *Candida pulcherrima*), and *Candida stellata* are thought to be the principal yeasts of grapes. In some studies, *Hanseniaspora* was reported to be the dominant species **(Beltran et al., 2002; Combina et al., 2005; Hierro et al., 2006)**, while *Candida* assumed to be widespread as well **(Clemente-Jimenez et al., 2004)**. Majority of *Candida stellata* isolates from wine are actually *Candida zemplinina* **(Csoma and Sipiczki, 2008)**.

Kačániová et al. (2018) isolated from the surface of grape berries different species of microorganisms. The most abundant G^- bacteria were *Stenotrophomonas maltophilia* and *Ignatzschineria indica*. Same results were found in the present study with wine grape berries. Within 22 different species of G^+ bacteria, *Bacillus endophyticus*, *Paenibacillus glucanolyticus*, *Paenibacillus lautus* and *Staphylococcus succinus* were the most isolated among bacteria *Rhodotorula mucilaginosa* was the most abundant among yeasts.

Kunová et al. (2018) found fungal counts ranged from 2.85 log cfu.g^{-1} in Cabernet Sauvignon to 4.83 log cfu.g^{-1} in Feteasca regala. After identification of 627 isolates of microscopic fungi, moulds belonged to genera *Alternaria* and *Penicillium* were the most widespread and were isolated from 100% of samples. *Alternaria* sp. was the most abundant fungal species in our study also. The high prevalence of *Aspergillus* (76.92%) and *Cladosporium* (76.92%) was found (Table 2).

Alternaria, *Cladosporium* and *Penicillium* were the most abundant moulds after identification of 1377 cultures of microscopic fungi isolates **Felsöciová et al. (2017)**. The identified prevalence found was similar to our results (100%). The higher prevalence was detected for *Fusarium* (100%), *Epicoccum*, *Rhizopus* (87.5%), *Botrytis*, *Aspergillus* (75%) and *Mucor* (62.5%). Different fungal genera with higher prevalence in comparison with our study were identified. **Kántor et al. (2017)** found 11 genera of G^- (11%), 11 of G^+ (27%) bacteria and nine genera of yeasts (62%) among 200 isolates of 19 Slovak grape samples. The most frequently isolated G^- bacteria were *Acinetobacter* (22%), *Pseudomonas* (22%) and *Sphingomonas* (13%). The most common genera of G^+ bacteria were *Bacillus* (20%), *Lactobacillus* (19%), *Leuconostoc* and *Staphylococcus* (11%). The most common yeasts genera were *Hanseniaspora* (37%), *Metschnikowia* (31%), and *Rhodotorula* (10%). Our results on diversity of microbial species in grape samples corresponded to **Kántor et al. (2017)** results.

Similar results were described in **Kántor et al. (2016)** study, who studied similar grape wine varieties as sampled in our study. The most dominant species was *Saccharomyces cerevisiae* isolated from all 15 new wine samples, that was a very good wine quality indicator. Altogether, seven different *S. cerevisiae* strains were identified with mass spectrometry, the second most common species was *Kloeckera apiculata (Hanseniaspora uvarum)* found in seven new wine samples. Also, other non – *Saccharomyces* yeasts such as *Metschnikowia pulcherrima*, *Pichia occidentalis* and *Pichia kluyveri* were identified.

CONCLUSION

Natural microflora of grape berries is very diverse. In our study, the bacteria were the most distributed in comparison with other groups of microorganisms. The highest bacterial counts were found in Palava grape variety, followed by Welschriesling. In our study, 5 different yeast, 9 moulds and 14 bacteria species on grape berries were identified.

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Acknowledgments:

This work has been supported by the grant of the European Union project no. 26220220180: Building Research Centre "AgroBioTech" and the grant of Slovak Research and Development Agency No. VEGA 1/0411/17.

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