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DETECTION OF MICROBIOTA IN THE VINEYARDS OF THE TOKAJ WINE REGION

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ABSTRACT

Tokaj is an important Central European wine-growing area with controlled planting and authorized varieties of white vines. This area has a specific microflora composition which changes based on its climate dependence, as well as during the fermentation process of wine production. Therefore, the aim of this study was, by culture examination of the samples, to detect the microbiota of soil, leaves, berries and fermentation must from two vineyards from the Slovak part of Tokaj. The highest total viable count (5.60 ±0.01 log cfu.g^{-1}) and the highest total yeast and mould count (4.32 ±0.01 log cfu.g^{-1}) in soil samples were recorded in vineyard Berecký. The highest total viable count in soil samples (6.71 ±0.01 log cfu.g^{-1}) was confirmed by examination of samples originating from the vineyard of Čierna Hora. When determining the total yeast and mould count, the highest numbers were recorded in the must samples (4.15 ±0.01 log cfu.mL^{-1}). Lactic acid bacteria were collected in samples from both vineyards, only in very low numbers. Overall, statistically significant differences (p <0.001) were detected by comparing the microbiota of the samples taken from the Berecký and Čierna Hora vineyards. The specific characterisation and identification of yeast was carried out using ITS-PCR-RFLP methods. The analysis confirmed the presence of yeasts of *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Candida parapsilosis* and *Candida tenuis* and their subsequent transfer to the must at varying percentages.

Keywords: microbiota wines; terroir; ITS-PCR-RFLP; yeast

INTRODUCTION

In the countries with the highest wine production, the microflora of the oenological process is regularly monitored for variety, climatic conditions and geographical location **(Piknová and Jankura, 2019)**. Tokaj is an important Central European wine region located in the Bodrog river basin. The uniqueness of the Tokaj region is that it extends on the territory of two independent states - Hungary and Slovakia. The Slovak part consists of vineyards in the cadastral territory of 7 wine-growing villages (Bara, Čerhov, Černochov, Malá Tŕňa, Slovenské Nové Mesto, Veľká Tŕňa and Viničky). Planting is controlled and only white varieties of vine are allowed, namely Furmint, Lipovina and Yellow Muscat **(Furdíková, Kakaš and Malík, 2015)**.

The quality of Tokaj wines is influenced by several factors. Soil resulting from weathered ryoliths has an impact. The high potassium content of these rocks has a positive effect on the vine, as it is increasingly required during the growing season. Another important and specific factor is the location of Tokaj vineyards with positive climatic conditions **(Farkaš, 2002)**. These factors also have a significant impact on the biogeography of microorganisms (including yeast) in the ecosystems **(Nedomová et al., 2016).** Yeasts (especially *Saccharomyces* spp.), which are the most important group of microorganisms in wine production, also have a significant impact on quality. The population of non-Saccharomyces species decreases in fermentation processes and the wine yeast *Saccharomyces cerevisiae* completes the fermentation. The ability of *S. cerevisiae* to replace non-*Saccharomyces* species is associated with its higher fermentative power, alcohol tolerance and secretion of killer-like compounds **(Albergaria and Arneborg, 2016)**. During fermentation also occur to other genera and species of microorganisms, which ultimately positively affect the taste, smell the wine, but can also have a negative impact **(Fugelsang and Edwards 2007; Piknová et al., 2017)**. All these microorganisms can be found ecologically linked to the wine environment **(Spano and Torriani, 2016)**.

Molecular biological methods in yeast identification can reliably determine which species are found in a given wine-growing area as well as which species apply at a certain stage of fermentation and how they affect this process. From grapes and wine, many yeast genera have

been identified by molecular biological methods such: *Brettanomyces*, *Candida*, *Kloeckera*/*Hanseniaspora*, *Kluzveromyces*, *Metschnikowia*, *Pichia*, *Saccharomyces* and *Zygosaccharomyces* **(Bartowsky, 2017)**.

Scientific hypothesis

The Tokaj wine region has a specific composition of microbiota, which, however, changes due to its climatic and geographical dependence, as well as during the fermentation and wine production process. Therefore, we expect differences in the composition of microbiota in two different vineyards in the Tokaj wine region, as well as changes in microbiota due to soil and vines on the must's microflora.

MATERIAL AND METHODOLOGY

Soil, grape leaves, white grape berries and must, from the Lipovina variety, from two vineyards in Slovak part of the Tokaj wine region were taken to determine the diversity of vine and must microbiota. Samples were taken from the Berecký vineyard, located in the wine village of Veľká Tŕňa, and from the vineyard Čierna Hora, located in the wine village of Čerhov. Sampling took place from May to October 2018 as follows: sampling of soil and vine leaves in May (1st sampling), subsequent sampling of vine leaves (2nd sampling) and grape berry in August and the first half of October, must samples 24 hours after pressing, were taken.

Culture microbiological examination of samples

Total viable count (TVC)

A 10 g base suspension and a further ten-fold dilution were prepared from the 10 g samples. Inoculum of 1 mL was inoculated in parallel to sterile Petri dishes from three consecutive dilutions. The inoculum was flooded with agar medium Plate Count Agar (PCA) for at least 15 minutes (18 ±2 mL). After agar solidification in labeled Petri dishes, the inoculated broths were incubated in a thermostate at 30 ±1 °C for 72 hours. After the incubation period, colonies were counted in inoculated Petri dishes. The results were evaluated according to **STN EN ISO 4833-1:2014**.

Lactic acid bacteria count (LABC)

A basic suspension and ten-fold dilutions were prepared from the samples according to **STN EN ISO 6887-1:2017**. From three consecutive dilutions, 0.1 mL was spread onto the surface of De Man, Rogosa and Sharpe agar (MRS; Oxoid, UK) selective diagnostic medium. Samples were prepared and evaluated in parallel. Subsequent incubation was performed under anaerobic conditions to propagate mesophilic lactic acid bacteria. The inoculated plates were placed in an anaerostat and incubated at 37 °C for 48 hours. The Anaerobic environment was provided by the AnaeroGen (Oxoid, UK).

Total yeast and mould count (TYMC)

The determination of the number of microscopic fungi and yeast was performed according to a standard procedure **(STN ISO 21527-1:2010)**. Three ten-fold consecutive dilutions were spread on the surface of Dichloran Rose Bengal Chloramphenicol (DRBC; HiMedia, India) agar medium at 0.1 mL and incubated for 5 – 8 days at 25 °C. Solitary yeast colonies taken sterile from the surface of DRBC agar were used for further studies.

Identification of yeast by ITS-PCR-RFLP

To obtain pure and concentrated DNA from yeast, a column isolation kit for the isolation of yeast DNA, NucleoBond® AXG Columns 20 (Macherey-Nagel GmbH & Co. KG, Germany) was used. DNA purity and concentration was detected using a BioSpec nanometer spectrometer (Shimadzu, Austria). The obtained supernatant was used as a DNA source in PCR reactions. The rRNA gene region of interest was amplified in a Thermal Cycler (Techne, Cambridge, UK). Primers used to amplify a given ITS region, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), were synthesized and used according to **(White et al., 1990)**. The PCR reaction was carried out in the following steps: initial denaturation at 95 °C/5 minutes, followed by 30 cycles of denaturation at 95 °C for 1 minute, annealing at 53 °C/2 minutes and extension at 72 °C/2 minutes, and final extension was performed at 72 °C for 10 minutes. The PCR products were sequenced by the Sanger method (GATC Biotech AG, Germany) and the subsequently obtained strains of those that were being studied were submitted to the GenBank-EMBL database for homology to the sequences available in the GenBank-EMBL database using the BLAST program (NCBI software package).

After evaluation, for accurate species identification, PCR products were digested with restriction endonucleases *Hha*I, *HaeIII* and *HinfI* (New England BioLabs®inc., USA), according to the supplier's instructions. The size of PCR products and restriction fragments was determined based on their mobility in agarose gels, in comparison to the 50 bp standard (Sigma-Aldrich, USA). PCR products and restriction fragments were visualized by UV transillumination using Mini Bis Pro® (DNR Bio-Imaging Systems Ltd., Israel).

Statistic analysis

Data analysis was carried out with R – statistics software **(R Core Team. R: 2019)**. A two-way analysis of variance (ANOVA) and Tukey test for multiple comparison of means with a confidence interval set at 95% was conducted according to **Semjon et al. (2018)**. The differences between the vineyard location and the wine processing phase were set as the main factors.

RESULTS AND DISCUSSION

Previous studies of grapes and grape musts microflora revealed valuable indigenous yeast strains, which could serve as the contributors to the regional character of wines specific to different winemaking regions **(Varela and Borneman, 2016; Raymond Eder et al., 2017)**.

Table 1 Culture microbiological examination of samples.

	Vineyard		Two-way ANOVA table (*p*-value)		
	Berecký (log cfu.g^{-1})	**Čierna hora (log cfu.g^{-1})**			
Variable	TYMC		**Vineyard (V)**	**Processing (P)**	**Interaction (V x P)**
Soil	4.32 ±0.01^{Ab}	3.82 ±0.01^{Bb}	<0.001	<0.001	<0.001
Leaves first sampling	2.81±0.01^{Bd}	3.3 ±0.01^{Ad}			
Leaves second sampling	4.78 ±0.01^{Aa}	3.64 ±0.01^{Bc}			
Grape berries	3.17 ±0.02^{Ac}	2.61 ±0.01^{Be}			
The must	3.17 ±0.02*^{Bc}	4.15 ±0.01*^{Aa}			
Variable	**TVC**				
Soil	5.6 ±0.01^{Ba}	6.71 ±0.01^{Aa}	<0.001	<0.001	<0.001
Leaves first sampling	4.18 ±0.01^{Ad}	4.15 ±0.01^{Be}			
Leaves second sampling	5.15 ±0.01^{Ab}	4.23 ±0.01^{Bc}			
Grape berries	4.2 ±0.01^{Ac}	4.18 ±0.01^{Bd}			
The must	4.2 ±0.01*^{Bc}	5.18 ±0.01*^{Ab}			
Variable	**LABC**				
Soil	-	-	ns.	ns.	ns.
Leaves first sampling	-	-			
Leaves second sampling	<3.00 ±0.00^{Aa}	<3.00 ±0.00^{Aa}			
Grape berries	-	-			
The must	<4.00 ±0.00*^{Aa}	<4.00 ±0.00*^{Aa}			

Note: V - the main effect of different Vineyard location; P - the main effect of processing phase of the vine production; V x P - interaction effect between the vineyard location and processing phase; ns. - not significant (p >0.05); $^{A–B}$ - in a column means (Vineyard) without a common superscript letter differ (p <0.05); $^{a–e}$ - in a row means (Processing) without a common superscript letter differ (p <0.05); * log cfu.mL^{-1}.

According to **Baroň (2017)**, the soil in the vineyard serves as a reservoir of microorganisms - yeast and bacteria. These are influenced by different selection pressures during the year, reflecting the composition of the soil microclimate, or the given terroir.

These selection pressures change the species representation of microorganisms that come from roots, trunk, leaves to the grapes and affect the microflora of the grapes during the season, and this therefore affects the wine.

To determine the differences in the microflora of soil, vines and musts from different vineyards of the Tokaj wine region, we used soil, vine leaves, grape berries and musts samples. Culture microbiological examination of the samples determined TVC, LABC and TYMC.

As shown in Table 1, the highest TVC was recorded in soil samples (5.60 ±0.01 log cfu.g^{-1}) and vine leaves at the second sampling (5.15 ±0.01 log cfu.g^{-1}). These results also correlate with yeast and fungi counts, where the TYMC also recorded in soil samples of 4.32 ±0.01 log cfu.g^{-1} and in vine leaf samples taken at the second harvest 4.78 ±0.01 log cfu.g^{-1}. LABC were detected only in very low numbers, namely in leaf samples (first sampling) and must collected in October.

Examination of samples originating from the vineyard of Čierna Hora confirmed the highest TVC in soil samples of 6.71 ±0.01 log cfu.g^{-1} and must 5.18 ±0.01 log cfu.mL^{-1}. When determining TYMC, the highest numbers were recorded only in the must samples (4.15 ±0.01 log cfu.mL^{-1}). In contrast, the lowest TYMC were recorded in grape berries (2.61 ±0.01 log cfu.mL^{-1}). The presence of LABC was confirmed in must and leaf samples (first sampling), but in very low numbers (Table 1). Similar to **Kačániová et al. (2019)**, which confirmed LABC counts in the must ranged from 0.48 to 2.06 log cfu.mL^{-1}.

Overall, statistically significant differences (p <0.001; Table 1) were detected by comparing the microbiota of samples taken from the vineyards of Berecký and Čierna Hora originating in the same wine-growing region.

After the cultivation examination of samples from the Tokaj wine-growing region, the team was, in turn, able to proceed with species identification of yeasts isolated on the surface of the DRBC agar medium.

Yeast DNA isolation was performed according to the commercially available NucleoBond® AXG Columns 20 kit. Isolation was followed by amplification of the ITS region of the rDNA using non-specific primers ITS1 and ITS4. Visualization of the resulting PCR products contained 450 bp - 880 bp fragments in the agarose gel. Individual PCR products were subjected to sequencing and subsequent comparison of the nucleotide sequence with the GenBank-EMBL database. Accordingly, PCR products

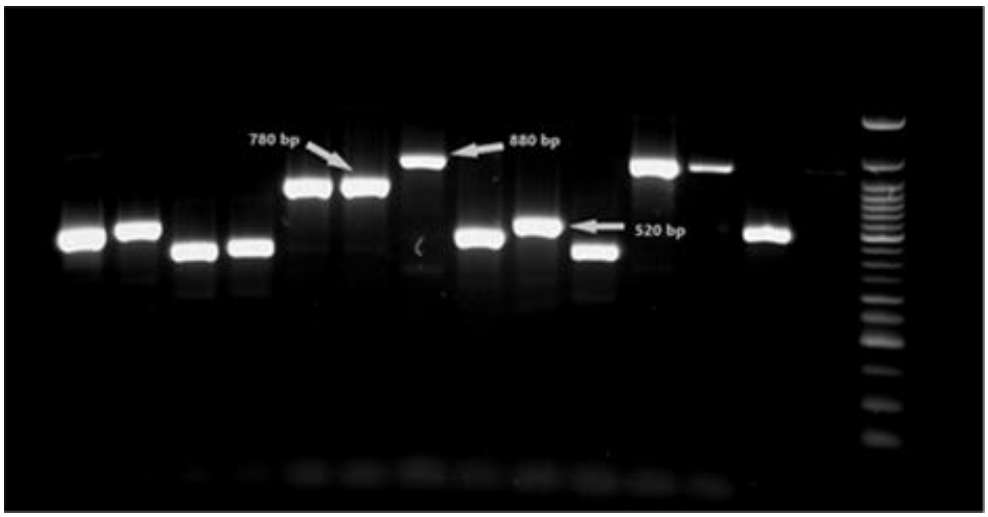
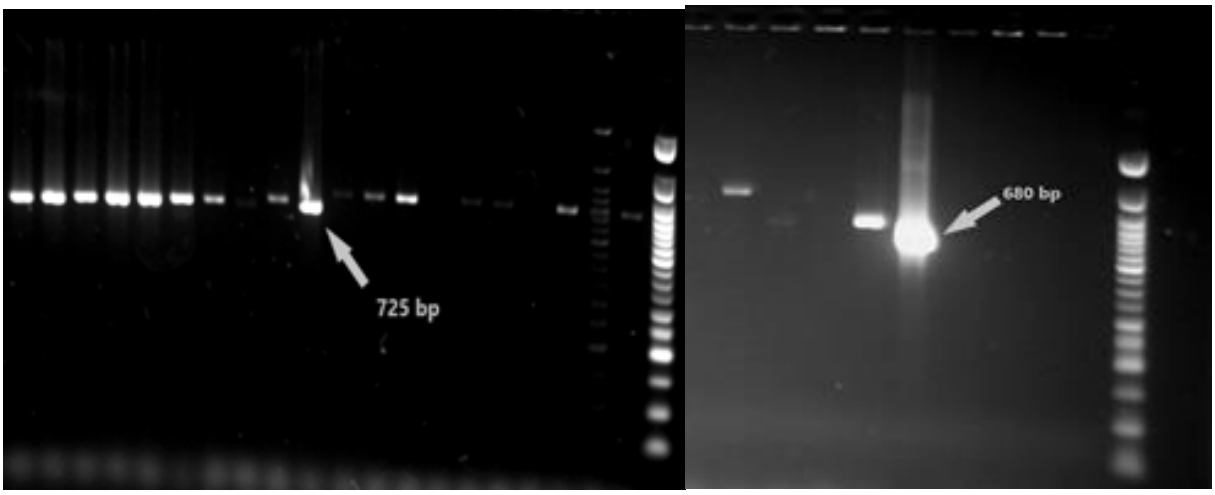


Figure 1 Visualization of PCR products in species *Saccharomyces* spp. 880 bp, *Zygosaccharomyces* spp. 780 bp, *Candida* spp. 520 bp.



A B

Figure 2 Visualization of PCR products in genus: *Zygosaccharomyces* 725 bp (A), *Candida* 680 bp (B).

of 880 bp size corresponded to yeasts of the genus *Saccharomyces*. PCR products of 780 bp and 725 bp corresponded to yeasts of the genus *Zygosaccharomyces* and fragments of 680 bp and 520 bp corresponded to the yeasts of *Candida* (Figure 1 and 2).

PCR products of smaller size (450 bp) were considered to be non-specific products that did not show relevant homology to any sequence in the GenBank-EMBL database, therefore such isolates were excluded from the study. Similarly, **Kačániová et al. (2018)** confirmed the presence of yeasts of the genus *Sachcaromyces* and *Candida* in grapes berries using molecular methods.

After the initial identification at the genus level, the individual PCR products were digested with restriction endonucleases *HhaI*, *HaeIII* and *HinfI* into fragments of different sizes, which generated species-specific restriction profiles (Figure 3 and 4). The restriction profiles of the yeast reference strains (CCM8224, CCM8239, CCM8260, CCM8191) were used to verify the correct yeast species, whereby identification took place via ITS-PCR-RFLP method. As shown in Figure 3 and 4, the restriction profiles of the individual yeast species differ from each other. This is confirmed by the study of **Sadel (2016)**, which also identified various types of yeast from grape must by ITS-PCR-RFLP.

After yeast species identification using the ITS-PCR-RFLP method, the percentages of individual yeast species in the soil, vine and must samples be originating from the Berecký and Čierna Hora vineyards were calculated.

As shown in Table 2, the species distribution of yeast varied in individual samples taken from the Berecký vineyards. In soil samples, the largest proportion of yeasts of the species *Zygosaccharomyces bailii* (90%) was confirmed. This type of yeast was detected at a lower percentage in other samples and this type of yeast was not detected in must. In contrast, *Zygosaccharomyces rouxii* was detected in leaf samples (May and August) and grape berries. The highest percentage was found in samples of vine leaves taken in May (57%).

The genus *Zygosaccharomyces* comprises some of the most feared species in the industries of high sugar and high acidic food products. *Zygosaccharomyces rouxii* is the microbe has the ability to live in environments of extreme salinity or saccharinity, and spoil your food, yet they can also facilitate the production of the subtle and nuanced characteristic flavors we appreciate so much in soy sauce

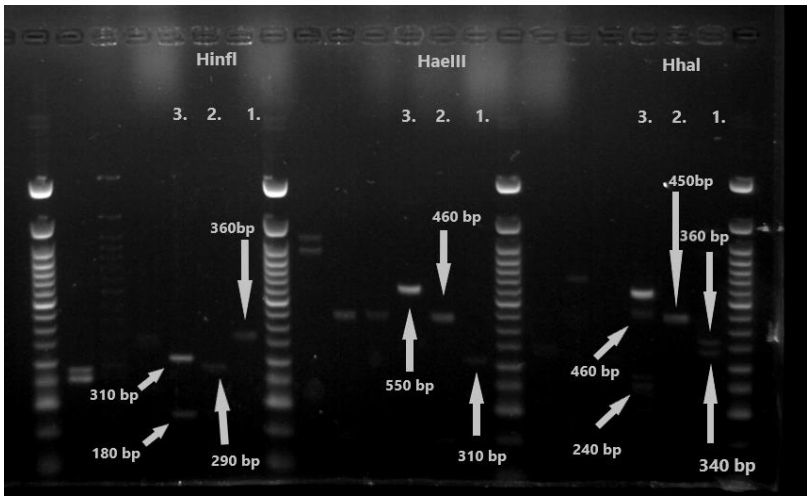
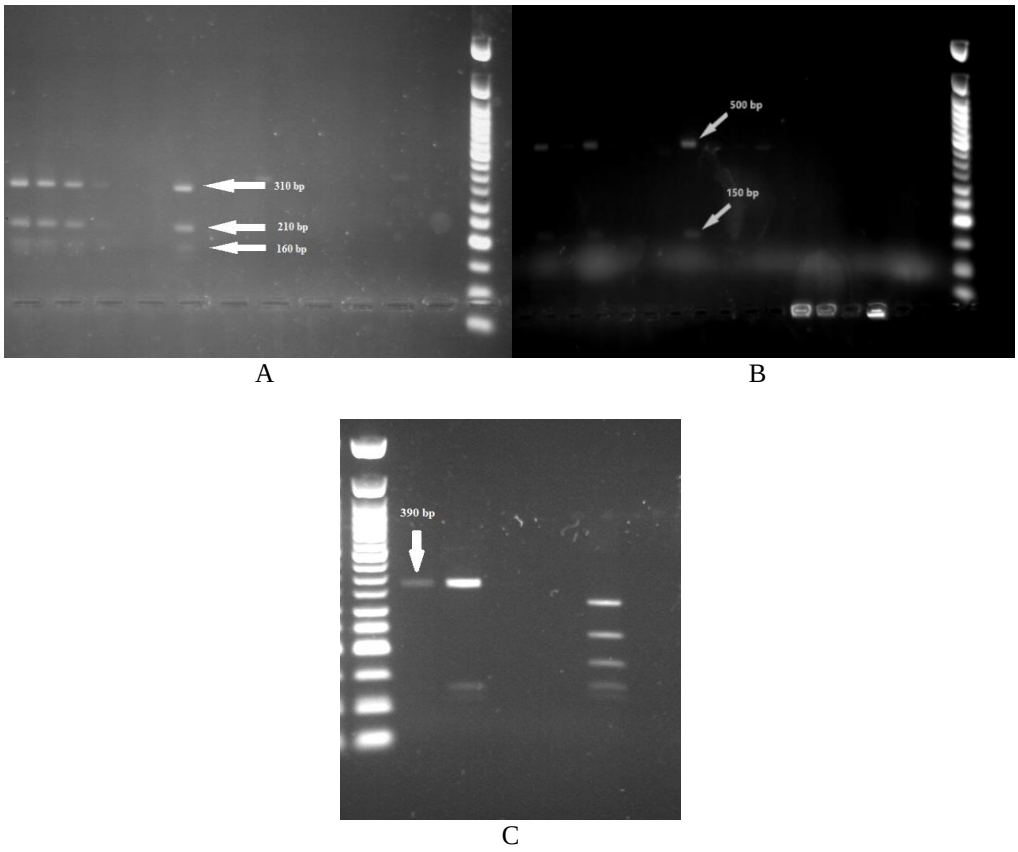


Figure 3 Restriction profiles of species: 1. *Saccharomyces cerevisiae*, 2. *Zygosaccharomyces rouxii*, 3. *Zygosaccharomyces bailii*



A B

C

Figure 4 Digestion of the PCR product with the restriction endonuclease *HinfI* (A), *HaeIII* (B) and *HhaI* (C) of *Candida tenuis*.

and other fermented foods **(Maffezzoli, 2017)**. On the other hand, *Zygosaccharomyces bailii* is notorious for its resistance to low pH, high concentration of organic acids, including preservatives, and possesses a rather high osmotolerance.

It causes off-flavours and vigorous alcoholic fermentation with abundant gas formation **(Rossi et al., 2010)**. It is the most problematic species for wines, with respect to its activities, which lead to visible sediment formation, cloudiness, or haziness in dry wines, and refermentation in sweet wines. Given the visual nature of the spoiling effect, it is of greater concern in white wines **(Fugelsang and Edwards, 2007; Barata, Malfeito-Ferreira and Loureiro, 2012)**.

Another species detected in the samples taken from the Berecký vineyard was *Saccharomyces cerevisiae*. Like *Zygosaccharomyces bailii*, it was detected in all samples of the Berecký vineyard. *Candida tenuis* and *Candida parapsilosis* species were also detected in vine leaves, berries and must. The percentages for both species varied significantly between samples (Table 2).

The same yeast species were detected in the samples of soil, vine leaves, berries and must taken from the vineyards of Čierna Hora as in the samples from the

Table 2 The percentage of yeast from the vineyard Berecký and Čierna Hora.

	Vineyard Berecký	Vineyard Čierna Hora
Samples	**Yeast species**	
Soil		
	90% *Zygosaccharomyces bailii*	90% *Zygosaccharomyces bailii*
	10% *Saccharomyces cerevisiae*	10% *Saccharomyces cerevisiae*
Leaves		
first sampling	57% *Zygosaccharomyces rouxii*	42% *Candida tenuis*
	29% *Candida tenuis*	33% *Zygosaccharomyces bailii*
	14% *Saccharomyces cerevisiae*	17% *Zygosaccharomyces rouxii*
		8% *Saccharomyces cerevisiae*
Leaves		
second sampling	55% *Candida tenuis*	80% *Candida tenuis*
	18% *Zygosaccharomyces bailii*	20% *Saccharomyces cerevisiae*
	9% *Zygosaccharomyces rouxii*	
	9% *Saccharomyces cerevisiae*	
	9% *Candida parapsilosis*	
Grape berries		
	32% *Candida parapsilosis*	54% *Candida tenuis*
	27% *Zygosaccharomyces bailii*	33% *Zygosaccharomyces rouxii*
	17% *Zygosaccharomyces rouxii*	13% *Saccharomyces cerevisiae*
	17% *Candida tenuis*	
	7% *Saccharomyces cerevisiae*	
The must		
	53% *Saccharomyces cerevisiae* 37% *Candida tenuis*	80% *Saccharomyces cerevisiae*
	10% *Candida parapsilosis*	20% *Candida tenuis*

Berecký vineyards, but at different percentages (Table 2). One of the yeast species detected was *Zygosaccharomyces bailii*. Its presence was confirmed in soil samples (90%) and vine leaf samples taken in May (33%). In contrast, the species *Zygosaccharomyces rouxii* was detected only in samples of vine leaves (harvest in May) and grape berries with the same percentage (33%).

Another yeast species detected was *Saccharomyces cerevisiae*, which was present in all percentages of samples from the vineyards of Čierna Hora. Only *Candida tenuis* was detected from yeasts of the genus *Candida*, namely samples of vine leaves, grapes and must (Table 2).

According to **Knight et al. (2015)** wine shows the strongest geographical signatures of all agricultural products and is therefore an excellent model for evaluation with respect to what extent it is affected by microbial terroir. Microorganisms, predominantly yeast, can significantly affect the "phenotype" of wine primarily by affecting the health and development of the vine and hence the quality of the wine. Also, their action during fermentation creates a number of secondary metabolites, including volatile compounds that have an impact on the flavor and aroma of wine. As **Fleet (2008)** states, microorganisms can very easily switch from soil to berries, changing the overall composition of the microflora. An important factor is the health of the berries because the microflora of healthy and rotten, damaged or bitten berries is significantly different. Also, insects greatly affect the diversity of yeast species, as they contribute to the transfer of different species of microorganisms from one environment to another. As reported by **Kántor et al. (2017)**, the species representation of yeasts on grape berries also depends on factors such as temperature, soil, rain, pesticide treatment and grape varieties. In the study, he identified a total of 65 bacteria and yeast species from 19 samples of Slovak grapes. A total of 123 yeast isolates were identified, mainly from the *Saccharomycetaceae* family (45%). **Kántor et al. (2017)** dealt with the microflora of grape berries, and the following three species were detected by *Candida* yeasts: *Candida saitoana*, *Candida magnolia* and *Candida parapsilosis*.

According to **Jackson (2008)**, the most common yeast species on ripe grapes are *Kloeckera apiculata*. Other yeasts that were also isolated from grapes include the genera *Brettanomyces*, *Candida*, *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Metschnikowia*, *Nadsonia*, *Pichia*, *Saccharomyces* and *Torulopsis*. In our study, however, only a total of 5 yeast species have been identified, which may be due to adverse climatic events between May and October 2018, whereby this period was characterized by below-average rainfall as well as above-average air temperatures **(SHMU, 2018)**. Such climatic conditions do not support the proper development of

microbiota on berries and vine leaves, as our findings suggest.

Saccharomyces cerevisiae (53%), to a lesser extent *Candida tenuis* and *Candida parapsilosis*, were detected in the must from Berecký vineyards. Only species of *Saccharomyces cerevisiae* (80%) and to a lesser extent *Candida tenuis* were detected in the vine originating in Čierna Hora (Table 2).

Despite the fact that *Candida* belongs to skin-forming undesirable microorganisms in wine ripening, *Candida tenuis* is the main producer of gluconic acid, which is found mainly in the must in the amount of 100 – 300 $mg.L^{-1}$. In musts of botrytic grapes the concentration may be increased to 6.0 $g.L^{-1}$. This causes the characteristic sensory properties of the mature wine. This species is found only sporadically in about 0.25% of total wine production **(Pandey et al., 2016)**.

Similarly, *Candida parapsilosis* appeared at a low frequency, in comparison to the total number of yeasts isolated from both musts, but **González et al. (2007)** found a large percentage (18.5%) of these yeasts at Agustín Díaz winery, thus, they may be responsible for distinctive and interesting wine properties. **Sipiczki et al. (2001)** studied the diversity of yeast microflora in spontaneously fermented wines in Tokaj. They found that fermentation began with a mixed population of yeast species, but on day 4, 59% of YPGA colonies belonged to *Saccharomyces* spp. and from day 8 already 98% of *Saccharomyces* spp. In their study, **Ženišová et al. (2014)** was also involved in mapping the diversity of wine yeasts and moulds in the Small Carpathian Wine Region. Genotypic identification was performed using the real-time PCR method. They have identified the presence of various yeast species, including *Saccharomyces cerevisiae*.

CONCLUSION

The work focused on finding differences in microbiota of soil, vine leaves, grape berries and must from two vineyards of the Tokaj wine region. Simultaneously, by means of ITS-PCR-RFLP method, yeast species representation in individual samples was found. The following species were identified: *Zygosaccharomyces rouxii*, *Zygosaccharomyces bailii*, *Candida tenuis*, *Saccharomyces cerevisiae*, *Candida parapsilosis*. The total microbiota as well as the species representation of yeast in the samples from the vineyards examined varied in percentage. However, since these vineyards are located in nearby locations, the second representation of yeast was similar. In addition, differentiation in the microbiote during the wine production process was confirmed. Yeast, especially non-*Saccharomyces*, has been confirmed in soil, leaves, and vine berries, but the presence of the genus *Saccharomyces* has already predominated in the musts.

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