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THE DIVERSITY OF FUNGAL POPULATION FROM GRAPE HARVEST TO YOUNG WINE IN SMALL CARPATHIAN WINE REGION

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ABSTRACT

The study aimed to identify the filamentous fungi and yeast mycobiota found on the surface and in grapes, grape must, and wine obtained from four red grape varieties: Alibernet, Dornfelder, Blue Frankish, Cabernet Sauvignon, and four white grape varieties: Green Veltliner, Rheinriesling, Pinot Blanc, Sauvignon. Grapes from vineyard Vrbové located in southwestern Slovakia were used for the research in 2020. The identification of filamentous fungi was performed using the macroscopic and microscopic observations and yeasts were identified by MALDI-TOF Mass Spectrometer. A total of 642 isolates were obtained. Grapes were rich in diversity of filamentous fungi (13 genera) and must on yeasts (8 genera). *Penicillium, Botrytis,* and *Hanseniaspora uvarum* were identified in both grapes and must. Three of the fungal genera identified by conventional or molecular techniques from the surface of red grape varieties were predominant: *Alternaria* (26%), *Botrytis* (21%), and *Issatchenkia terricola* (13%), two from endogenous mycobiota: *Hanseniaspora uvarum* (45%) and *Botrytis* (17%), four from the surface of white grape varieties: *Penicillium* (25%), *Botrytis* (21%), *Alternaria* (16%) and *Hanseniaspora uvarum* (15%), and three from endogenous mycobiota: *Botrytis* (44%), *Hanseniaspora uvarum* (23%) and *Alternaria* (20%). *Saccharomyces cerevisiae, Candida krusei, C. utilis,* and *Cryptococcus neoformans* were identified only in wine.

Keywords: yeast; filamentous fungi; *Penicillium;* must; mass spectrometry

INTRODUCTION

Microfungi are ubiquitous microorganisms in the environment. If certain physical conditions, such as moisture level, temperature, and the presence of organic and inorganic substrates, are met in fungi, they can easily proliferate **(Andersen and Thrane, 2006)**. Wine grapes are no exception. Wine grapes (*Vitis vinifera,* L.) are an economically and culturally important agricultural commodity for which microbial activity plays key roles in grape and wine production and quality **(Barata, Malfeito-Ferreira and Loureiro, 2012; Swiegers et al., 2005)**. The grapevine harbors complex and diverse microbiota, such as bacteria, filamentous fungi, and yeasts **(Barata, Malfeito-Ferreira and Loureiro, 2012; Liu and Howell, 2020; Stefanini and Cavalieri, 2018)**, which substantially modulate vine health, growth, and crop productivity **(Gilbert, van der Lelie and Zarraonaindia, 2014; Müller et al., 2016)**.

Recently, winemakers have started to realize the potential contribution offered by the indigenous microbial population in producing a wine closely associated with geographical origin **(Tristezza et al., 2013)**. The geographical area, together with cultivar, climate, and vintage, is the major determinant for the microbiota of must at the beginning of the fermentation process **(Bokulich et al., 2014)**. Grapevine associated microbiota can be transferred to the grape must/juice and influence the production of secondary metabolites on wine composition, aroma, flavor, and quality **(Barata, Malfeito-Ferreira and Loureiro, 2012; Ciani et al., 2010; Calabretti et al., 2012; Morrison-Whittle and Goddard, 2018)**. Fine control of the composition of must microbiota is of paramount importance for the quality of the final product, since different components of must, the microbiota can contribute in contrasting ways to the aroma of the final product, giving either pleasant or undesirable aromatic notes to the wine. It is well known that several factors related to grape juice (i.e. grape composition and chemical characteristics, ethanol accumulation, and temperature) can affect the kinetics of yeast growth **(Fleet and Heard, 1993; Bisson, 1999; Zott et al., 2008)**. Autochthonous fermentation (also known as native or inoculated) is believed to display more complexity in aroma and mouthfeel characters than those conducted with a less rich and complex microbiota **(Boynton and Greig, 2016)**. In contrast, in many wine production regions, the grape juice

or must is immediately inoculated with a commercial strain of *Saccharomyces cerevisiae* **(Bisson, Joseph and Domizio, 2017)**. During fermentation, the microbiota can be affected by both microbial and chemical–physical factors. Some fungal species can either carry out an antimicrobial activity against certain other species/strains **(Oro, Ciani and Comitini, 2014)** or have a positive effect on the growth of other species **(Contreras, Curtin and Varela, 2014)**. Even though a part of this early stage microbiota does not survive the stressful conditions of late must fermentation, it still plays a role in shaping the entire process **(Heard and Fleet, 1988)**. Wine is the end product of the fermentative activity of yeast and bacteria. The microbiota of grape juice fermentation can vary significantly as over 40 genera and 100 different species of yeast have been isolated from grapes or wine **(Bisson, Joseph and Domizio, 2017)**. This paper reports on work to isolate and identify the filamentous fungi and yeast mycobiota from grape harvest from the Small Carpathian wine region of Slovakia to young wine.

Scientific hypothesis

Most fermented products are generated by a mixture of microbes. Wine is no exception. Substantial yeast, fungal and bacterial biodiversity is observed on grapes, and in both must and wine.

MATERIAL AND METHODOLOGY

Samples

Grape sampling

The grape samples were harvested during the 2020 vintage, from Sabo winery, Vrbové in the Small Carpathian wine region. The study area was described previously **(Felšöciová and Kačániová, 2019a)**.

Table 1 Wine grape varieties used in the study, date of harvest, sugar content, and selected yeast.

Grape variety	Date of harvest	Sugar content	Winemaking yeast
Alibernet	20.10.2020	19 °NM	Laffort, Actiflor rosé
Dornfelder	30.09.2020	19.5 °NM	reinoculated
Blue Frankish	20.10.2020	20 °NM	Laffort, Actiflor rosé
Cabernet Sauvignon	17.10.2020	20.5 °NM	Laffort, Actiflor rosé
Green Veltliner	10.10.2020	21 °NM	Laffort, delta
Rheinriesling	07.10.2020	20 °NM	Spontaneous
Pinot Blanc	03.10.2020	20 °NM	Mycoferm, CRU611
Sauvignon	24.09.2020	20 °NM	Laffort, X5

In total, 8 grape samples without visual signs of fungal invasion were collected (Table 1).

Four samples of red grapes (Alibernet, Dornfelder, Blue Frankish, Cabernet Sauvignon) and four samples of white grapes (Green Veltliner, Rheinriesling, Pinot Blanc, Sauvignon) were collected from multiple bunches of different grapevines, randomly distributed across the vineyard to assure the representativeness of the sampling. These samples were put into sterile plastic bags and transported to the laboratory chilled on ice and stored at – 20 °C until processing. Fresh grape must be prepared by crushing. Fermentation occurred in stainless steel tanks and was conducted with/without the addition of commercial yeasts (Table 1). Must and young wine of each wine grape variety were acquired and sampled in a winery, sent to the laboratory, and stored at 6 °C in the refrigerator.

Chemicals

DRBC medium (Dichloran Rose Bengal Chloramphenicol, MERCK, Germany), MEA (Malt extract agar) **(Samson et al., 2010)**, CYA (Czapek yeast agar) **(Samson et al., 2010)**, CREA (Creatine-Sucrose agar) **(Samson et al., 2010)**, YES (Yeast extract agar) **(Samson et al., 2010)**, formic acid (Sigma-Aldrich), acetonitrile (Sigma-Aldrich), matrix α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich).

Biological Material

Commercial yeasts: Laffort, Actiflor rosé, Laffort, delta, Mycoferm, CRU611, Laffort, X5.

Grapevine Material

Four samples of red grapes: Alibernet, Dornfelder, Blue Frankish, Cabernet Sauvignon and four samples of white grapes: Green Veltliner, Rheinriesling, Pinot Blanc, Sauvignon from Sabo winery, Vrbové in Small Carpathian wine region.

Instruments

Stomacher easyMix®, MALDI-TOF-MS Biotyper (Bruker Daltonics, Bremen, Germany).

Laboratory Methods

Plating methods with and without surface disinfection by the researcher **Magnoli et al. (2003)**, MALDI-TOF MS measurement by **Patel (2015)**.

Description of the Experiment

Mycological analysis

For each sample, the mycological diversity was analyzed at three stages: grapes must be obtained by the crushing of grapes and end of alcoholic fermentation as a young wine. The detection of fungi in grape samples was made by plating methods with and without surface disinfection. The individual samples were composed of 50 berries, plated on DRBC medium 7 – 8 berries per plate, and incubated for 5 – 7 days at 25 ±1 °C in the dark. Fungi were also isolated from the interiors of the grapes. Each grape surface (a total of 50 berries from each sample) was sterilized for 1 min in 1% NaClO and washed three times with sterile distilled water according to methods of **Magnoli et al. (2003)**, dried, plated onto DRBC, and incubated.

At a liquid stage, 200 mL of must and unfiltered wine were collected in sterile plastic bottles and stored at 6 ±1 °C in the refrigerator. Must sample in an amount of 20 mL were diluted with 180 mL of sterile physiological saline (0.85%) and shaken on a Stomacher easyMix®.

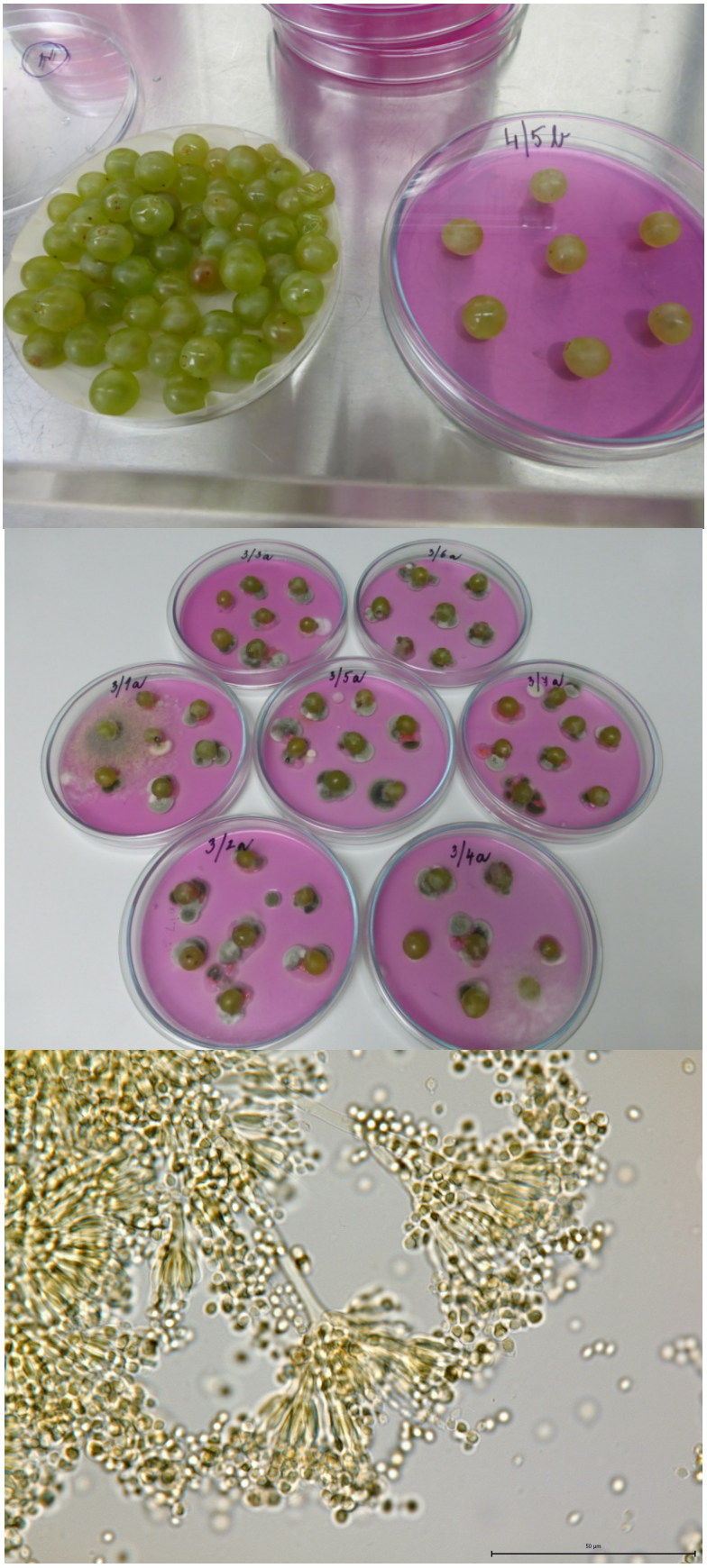

Figure 1 Direct plating of grapes onto DRBC, Petri dishes after incubation, identification of *Penicillium* sp.

Dilutions of 10^{-1} and 10^{-4} in the double were surface inoculated in the amount of 0.1 mL on MEA agar plates and cultivated at 25 ±1 °C in the dark. An undiluted sample of wine was also applied in an amount of 0.1 mL to MEA and the plates were incubated at 25 ±1 °C for 5 days.

The developing filamentous fungi were counted and identified based on macro and microscopic characteristics

using the following references: **Pitt and Hocking (2009)** and **Klich (2002)**. *Penicillium* spp. were picked off onto MEA, CYA, CREA, and YES to obtain pure cultures and identify further species. The identification of isolated strains was carried out according to special mycological literature **(Pitt and Hocking, 2009; Samson and Frisvad, 2004; Samson et al., 2002; Samson et al., 2010)**. Yeasts were identified by MALDI-TOF Mass Spectrometry.

MALDI-TOF MS measurement

The colony of yeast that was cultivated on Petri dish was transferred to Eppendorf microtubes containing 300 µL of ultrapure water and then 900 µL of ethanol (99%) was added. The sample was centrifuged for 2 minutes at 14 000 rpm and the supernatant was discarded. 30 µL of 70% formic acid (Sigma-Aldrich) and 30 µL of acetonitrile (Sigma-Aldrich) were added to the pellet and the pellet was resuspended thoroughly. The Eppendorf microtubes were centrifuged again at 14 000 rpm for 2 minutes. From the sample thus prepared, we applied 1 µL of the supernatant to a MALDI-TOF-MS target plate and allowed the sample to dry at laboratory temperature. From the prepared sample, 1 µL of the supernatant was applied to a MALDI-TOF-MS target plate and the sample was dried at laboratory temperature. After drying, 1 µL of a matrix α-cyano-4-hydroxycinnamic acid (10 $mg.mL^{-1}$, Sigma-Aldrich), was added to the surface of the sample. After crystallization at laboratory temperature, the target plate was placed into the ionization chamber of the mass spectrometer. The samples were processed by linear and positive mode MALDI-TOF MicroFlex LT/SH (Bruker Daltonics, Germany) in the range 200 – 2000 m/z.

Number of samples analyzed: we have analyzed 8 grape varieties, 8 musts and 8 young wines.

Number of repeated analyses: 4 times.

Number of experiment replication: 3 times.

Statistical Analysis

The obtained results were evaluated and expressed according to relative density (RD). The relative density (%) is defined as the percentage of isolates of the species or genus, occurring in the analyzed sample **(Guatam, Sharma, and Bhadauria, 2009)**. These values were calculated according to **González et al. (1999)** as follows:

$$RD\ (\%) = (ni/Ni) \times 100$$

Where:

ni –number of isolates of a species or genus; Ni – total number of isolated fungi.

RESULTS AND DISCUSSION

The overall mycological colonization of 4 tested red grape varieties from the surface and endogenous mycobiota of grapes, must and wine are summarized in Table 2. A total of 249 strains of microscopic fungi belonging to 10 genera of filamentous fungi and 10 genera of yeasts were identified. Isolation of fungi colonized surface grapes resulted in the collecting of 53 fungal isolates (39 isolates from filamentous fungi and 14 isolates from yeasts). Data in Table 2 also show that 10 filamentous fungal genera and 4 yeast genera were identified from surface grape samples. *Alternaria* was the most abundant occurring genus which recorded 14 isolates with a relative density of 26% (RD) of all the isolates found. *Botrytis* was the second predominant genus which recorded a relative density of 21%, followed by *Issatchenkia terricola* with 13% RD, *Mucor* with 7% RD, and *Hanseniaspora uvarum* together with *Aureobasidium pullulans* (6%, each) of all the fungi found. The remaining 8 genera were detected in less than 5%. *Alternaria* (26% RD), *Penicillium* (23% RD), *Rhizopus* (15% RD), and *Cladosporium* (11% RD) were the main components of the 3 red grape mycobiota (Alibernet, Cabernet Sauvignon, and Blue Frankish) of the Vrbovský subregion at harvest time 2019 **(Felšöciová, Kačániová, and Vrábel, 2020)**. *Alternaria* (87% RD) was still the main component of Alibernet, Cabernet Sauvignon, and Dornfelder grapes mycobiota from Vrbové in 2018 **(Felšöciová and Kačániová, 2019c)**. The same 3 red grape samples plus Blue Frankish collected in 2017 were analyzed for the occurrence of yeasts by **Kačániová et al. (2018)**. Within 39 isolates of yeasts, the most abundant was *Rhodotorula*. This genus we identified only from must. *Saccharomyces*, the major wine yeast is not ubiquitous on the ripening grape and if present, only constitutes very small fractions of the yeast communities **(Setati et al., 2013; Bokulich et al., 2014; Taylor et al., 2014)**. *Saccharomyces* strains are more frequently isolated from heavily damaged grapes **(Mortimer and Polsinelli, 1999)**, where the juice of the grape became accessible to the yeasts through the skin lesions. Consistent with these earlier observations, no *Saccharomyces* was found in this study among the colonies when the samples were plated directly on the agar medium. Samples belonging to red grape varieties Alibernet and Blue Portugal from Suchá nad Parnou showed different abundance from surface colonization **(Felšöciová and Kačániová, 2019b)**. The most abundant genera were *Cladosporium* (28% RD), *Alternaria, Epicoccum* (26%, each), and *Botrytis* (24% RD) in the harvest year 2016 and 2017 *Cladosporium* (24% RD), *Penicillium* (23% RD), *Alternaria* (21% RD) and *Epicoccum* (14% RD) of all the fungal isolates found. **Medina et al. (2005)** referred to the diversity of filamentous fungi isolated from muscat grape varieties grown in Spain. *Cladosporium* was the most common strain isolated from two blue varieties Garnacha and Monastrell (78.2% and 92.2%, respectively) of all isolates. **Abrunhosa et al. (2001**) reported that *Alternaria* and *Cladosporium* were more often isolated from red grape varieties than white, regardless of the vineyard in Portugal, which can not be confirmed from our study. *Cladosporium* was detected only once.

All the previous fungal genera except *Acremonium, Aspergillus, Dipodascus,* and *Issatchenkia* were also isolated from endogenous colonization. Nevertheless, the relative abundances varied.

Table 2 The occurrence and relative density of filamentous fungi and yeast identified from exogenous and endogenous mycobiota of 4 red grapes, must and wine from Small Carpathian wine region.

Taxa	grapes exo		grapes endo		must		wine		Total	RD (%)
Filamentous fungi	**No**	**RD (%)**	**No**	**RD (%)**	**No**	**RD (%)**	**No**	**RD (%)**		
Alternaria	14	26	3	4	-	-	-	-	17	7
Acremonium	1	2	-	-	-	-	-	-	1	<1
Arthrinium	1	2	1	1	-	-	-	-	2	<1
Aspergillus	2	4	-	-	-	-	-	-	2	<1
Botrytis	11	21	11	17	3	2	-	-	25	10
Cladosporium	1	2	2	3	-	-	-	-	3	1
Mucor	4	7	7	11	-	-	-	-	11	4
Mycelia sterilia	-	-	1	1	-	-	-	-	1	<1
Penicillium	2	4	1	1	72	57	-	-	75	30
P. crustosum	-	-	1	-	-	-	-	-	1	-
P. expansum	-	-	-	-	72	-	-	-	72	-
P. sp.	2	-	-	-		-	-	-	2	-
Rhizopus	1	2	1	1	-	-	-	-	2	<1
Trichoderma	2	4	1	1	-	-	-	-	3	1
Total of fil. fungi	**39**		**28**		**75**				**142**	
Yeast taxa										
Aureobasidium pullulans	3	6	6	9	-	-	-	-	9	4
Candida	-	-	-	-	6	5	2	67	8	3
C. catenulata	-	-	-	-	1	-	-	-	1	-
C. dubliniensis	-	-	-	-	1	-	-	-	1	-
C. krusei	-	-	-	-	2	-	2	-	4	-
C. oleophila	-	-	-	-	1	-	-	-	1	-
C. tropicalis	-	-	-	-	1	-	-	-	1	-
Cryptococcus	-	-	-	-	2	2	1	33	3	1
Cr. flavescens	-	-	-	-	1	-	-	-	1	-
Cr. neoformans	-	-	-	-	1	-	1	-	2	-
Dipodascus	1	2	-	-	-	-	-	-	1	<1
Hanseniaspora uvarum	3	6	30	45	22	17	-	-	55	22
Issatchenkia terricola	7	13	-	-	7	5	-	-	14	6
Metschnikowia pulcherrima	-	-	-	-	13	10	-	-	13	5
Pichia occidentalis	-	-	-	-	1	<1	-	-	1	<1
Rhodotorula	-	-	-	-	1	<1	-	-	1	<1
Saccharomyces cerevisiae	-	-	2	3	-	-	-	-	2	<1
Total of yeasts	**14**		**38**		**52**		**3**		**107**	
Total isolates	**53**		**66**		**127**		**3**		**249**	

Note: No – number of isolates; RD – relative density.

From endogenous colonization were reached 66 strains belonging to 8 genera and *Mycelia sterilia* of filamentous fungi (28 isolates) and 3 genera of yeasts (38 isolates). *Mycelia sterilia* is an unidentified microorganism – fungus without creation fruiting bodies. The most abundant genera were *Hanseniaspora uvarum* (45%), *Botrytis* (17%), and *Mucor* (11%) of all the isolates. The occurrence of *Botrytis,* as described **Felšöciová and Kačániová (2019b)**, was detected in 2% of the isolates from endogenous mycobiota in Alibernet and Blue Portugal in 2017 but in 2016 *Botrytis* was one of the most abundant genera (24% RD). The most abundant genera found by descending order except *Botrytis* were *Alternaria* (29% RD), and *Cladosporium* (23% RD) in 2016, and *Penicillium* (38% RD) and *Cladosporium* (29% RD) in 2017. *Penicillium* spp. in our red grape samples was generally low. Only *P. crustosum* was isolated. *Alternaria* (87% RD) was the most common genus not only in exogenous mycobiota but also in the endogenous mycobiota from Alibernet, Cabernet Sauvignon, and Dornfelder from Vrbové in 2018 **(Felšöciová and Kačániová, 2019c)**.

The highest number of yeasts was found from the must of red grape samples. A total of 52 strains of yeasts belonging to 7 genera were identified. The most abundant genera were *Hanseniaspora uvarum* (17% RD), *Metschnikowia pulcherrima* (10% RD), and *Candida* (5% RD) with 5 species of all the 127 fungi found. The apiculate yeasts usually predominate the early phase of fermentation and produce compounds, that enrich the aroma profile of the wine **(Zironi et al., 1993; Romano et al., 2003; Moreira et al., 2011; Sipiczki, 2016; Cioch et al., 2021)**. Pulcherrimin-producing *Metschnikowia* strains are common on ripe grapes. They are usually assigned to *M. pulcherrima* (*C. pulcherrima*) or less frequently to *M. fructicola* in the oenological literature **(Sipiczki, 2016)**. Species from the genus *Hanseniaspora, Candida, Pichia, Zygosaccharomyces,* and *Kluyveromyces* most desirably determine the diversity and complexity of the taste of wine **(Romano et al., 2003; Jolly, Varela and Pretorius, 2014)**. Most of them were identified from our samples of must. Must was riched on *Penicillium expansum* (57%). Except for *Penicillium expansum* (72 isolates), three

isolates of *Botrytis* (2%) were detected. Berries affected by *P. expansum* have an off-flavor and even a small amount of infected berries add a mouldy taste to the wine **(König and Fröhlich, 2017)**. **Felšöciová, Mašková and Kačániová (2018)** described 5 isolates of microfungi belonging to 3 genera of *Alternaria, Aspergillus* and *Cladosporium* and *Mycelia sterilia* from grape juice from red variety Dornfelder. Yeast counts in fresh grape juice were 2 x 10^5 CFU.mL^{-1}. It is considered that *Dekkera/Brettanomyces* are the most important wine spoilage microorganisms **(Bartowsky et al., 2003; Beneduce et al., 2004; Cocolin et al., 2004)**. In this study, *Dekkera/Brettanomyces bruxellensis* was not detected, which is in line with the study of **Suárez et al. (2007)**, who reported that this spoilage yeast is mainly present in winemaking equipment with deficient cleaning, and is opposed to the findings reported by **Renouf and Lonvaud-Funel (2007)**.

In red wine were not detected any filamentous fungi what confirmed in their study also **Felšöciová, Mašková and Kačániová (2018)**.

A low number of yeasts was recorded, namely *Candida krusei* (2 isolates) and *Cryptococcus neoformans* (1 isolate). According to **Felšöciová, Mašková and Kačániová (2018**) yeast at the end of fermentation slightly decreased on 5.7 x 10^4 CFU.mL^{-1}.

Fungal profiles of the various 4 white grape varieties, must and wine are summarized in Table 3. A total of 393

Table 3 The occurrence and relative density of filamentous fungi and yeast identified from exogenous and endogenous mycobiota of 4 white grapes, must and wine from Small Carpathian wine region.

Taxa	**grapes exo**		**grapes endo**		**must**		**wine**		**Total**	**RD (%)**
Filamentous fungi	**No**	**RD (%)**	**No**	**RD (%)**	**No**	**RD (%)**	**No**	**RD (%)**		
Alternaria	11	16	23	20	-	-	-	-	34	9
Arthrinium	1	1	-	-	-	-	-	-	1	<1
Asperillus	2	3	-	-	-	-	-	-	2	<1
Botrytis	14	21	50	44	10	5	-	-	74	19
Cladosporium	1	1	-	-	-	-	-	-	1	<1
Epicoccum	-	-	1	<1	-	-	-	-	1	<1
Fusarium	1	1	-	-	-	-	-	-	1	<1
Mucor	2	3	3	3	-	-	-	-	5	1
Mycelia sterilia	-	-	1	<1	-	-	-	-	1	<1
Penicillium	17	25	5	4	119	65	-	-	141	36
P. expansum	17	-	5	-	36	-	-	-	-	-
P. glabrum	-	-	-	-	83	-	-	-	-	-
Phoma	-	-	1	<1	-	-	-	-	1	<1
Rhizopus	5	7	1	<1	-	-	-	-	6	1
Total of fil. fungi	**54**		**85**		**129**				**268**	
Yeasts taxa										
Aureobasidium pullulans	1	1	1	<1	-	-	-	-	2	<1
Candida	-	-	-	-	15	8	1	5	16	4
C. catenulata	-	-	-	-	1	-	-	-	1	-
C. haemulonii	-	-	-	-	1	-	-	-	1	-
C. innocuum	-	-	-	-	1	-	-	-	1	-
C. krusei	-	-	-	-	2	-	-	-	2	-
C. lambica	-	-	-	-	1	-	-	-	1	-
C. lusitaniae	-	-	-	-	1	-	-	-	1	-
C. parapsilosis	-	-	-	-	1	-	-	-	1	-
C. tropicalis	-	-	-	-	6	-	-	-	6	-
C. utilis	-	-	-	-	-	-	1	-	1	-
C. valida	-	-	-	-	1	-	-	-	1	-
Cryptococcus	-	-	-	-	3	2	-	-	3	<1
Cr. flavescens	-	-	-	-	1	-	-	-	1	-
Cr.neoformans	-	-	-	-	2	-	-	-	2	-
Dipodascus	3	4	-	-	-	-	-	-	3	<1
Hanseniaspora uvarum	10	15	26	23	10	5	-	-	46	12
Kluyveromyces nonfermentans	-	-	-	-	1	<1	-	-	1	<1
Metschnikowia pulcherrima	-	-	1	<1	33	18	-	-	34	9
Pichia	-	-	-	-	2	<1	-	-	2	<1
Pichia kluyveri	-	-	-	-	1	-	-	-	1	-
Pichia occidentalis	-	-	-	-	1	-	-	-	1	-
Saccharomyces cerevisiae	-	-	-	-	-	-	18	95	18	5
Total of yeasts	**14**		**28**		**64**		**19**		**125**	
Total isolates	**68**		**113**		**193**		**19**		**393**	

Note: No – number of isolates, RD – relative density.

strains of microscopic fungi belonging to 20 genera and *Mycelia sterilia* were obtained from surface and endogenous mycobiota of grapes, must, and wine. The filamentous fungi population was richer than the yeast population from surface mycobiota. A total of 68 strains belonging to 12 genera were identified (9 from filamentous fungi and 3 from yeasts). *Penicillium expansum* was isolated in large amounts (25% RD), followed by *Botrytis* (21% RD), *Alternaria* (16% RD), and *Hanseniaspora uvarum* (15% RD) of all the isolates. Our results corroborate the findings of **Felšöciová, Kačániová and Vrábel (2020)** in which 26 % of isolates were *Penicillium* and 21% *Alternaria* from 9 white grapes from Vrbové during the harvest 2019. *Alternaria* was also one of the main fungal genera isolated from Tunisian grape berries **(Melki Ben Fredj et al., 2007**), Spanish grapes **(Bau et al., 2005; Medina et al., 2005)**, Moravian grapes **(Ostrý et al., 2007)**. Yeasts as *Sporobolomyces roseus, Cryptococcus albidus, Rhodotorula rubra,* and *Candida* were part of the natural microbiota of certain varieties of grapes in southern Spain **(De la Torre et al., 1999)**. In Egypt, **Haridy (1994)** found that the most common spoilage yeast of grapes was *Hanseniaspora valbyensis*. Also, *Hanseniaspora* species were reported as common yeast constituents on grapes **(Phister et al., 2007)** as confirmed by our results.

A total of 113 isolates of microscopic fungi belonging to 10 genera and *Mycelia sterilia* were obtained from endogenous mycobiota (7 from filamentous fungi and 3 from yeasts). The highest relative density was reached by the genera *Botrytis* (44%), *Hanseniaspora uvarum* (23%), and *Alternaria* (20%). *Penicillium expansum* (4%) contributed a small proportion of all fungi in comparison with exogenous colonization. Interestingly, *Botrytis* from the same grape samples was detected less than 1% in the harvest year 2018 **(Felšöciová and Kačániová, 2019c)**.

The microbial community present in the must before fermentation was rich, especially the diverse biodiversity of yeasts. Data in Table 3 show that 2 filamentous fungal genera and 6 yeast genera were identified. The dominant genus across the entire microfungi population was *Penicillium* (65%) and in a small proportion *Botrytis* (5%). Two *Penicillium* species, namely *P. glabrum* (83 isolates) and *P. expansum* (36 isolates) were identified. From the yeast population, the dominant species was *Metschnikowia pulcherrima* (18%), followed by *Candida* (8%) with 9 species and *Cryptococcus* (2%) with 2 species. *Pichia* and *Kluyveromyces* were identified as less than 1%. *Cladosporium* (75.5% RD) and *Penicillium* (10.2% RD) were present with high abundance in 47 must samples collected from five Slovakian wine regions, representing on average 75.5% and 10.2%, respectively of the fungal populations **(Felšöciová, 2016)**. The *Penicillium* genus was made up of *P. bilaiae, P. brevicompactum, P. citrinum, P. crustosum, P. expansum, P. funiculosum, P. griseofulvum, P. chrysogenum, P. polonicum,* and *P.* sp. **Barboráková et al. (2011)** obtained information about the mycobiota of Slovak origin wines during the production process in the year 2009. Altogether thirty-three samples from the production process of 5 species of white Slovak origin wines were mycologically analyzed. The spectrum of isolated penicilia consisted of twenty-one species: *Penicillium aurantiogriseum, P. brevicompactum, P. citreonigrum, P. citrinum, P. corylophilum, P. crustosum, P. decumbens, P. expansum, P. funiculosum, P. glabrum, P. griseofulvum, P. implicatum, P. oxalicum, P. paneum/carneum, P. pinophilum, P.polonicum, P. purpurogenum, P. restrictum, P. roqueforti, P. rubrum, and P. rugulosum*. **Pinto et al. (2015)** characterized and compared the diversity of the microbial communities during spontaneous wine fermentations from samples collected from six Portuguese wine regions. In general, the fungal populations of initial must were characterized by ubiquitous genera as *Aureobasidium, Rhodotorula, Hanseniaspora, Alternaria, Metschnikowia, Saccharomyces, Candida, Ramularia, Penicillium, Lewia, Filobasidiella, Leptosphaerulina,* and *Schizosaccharomyces*, forming the principal structure of the microbial populations. This is in line with the previous study reported by **Bokulich et al. (2014)**, where microorganisms as *Cladosporium* spp., *Aureobasidium pullulans, Hanseniaspora uvarum* were detected as the major eukaryotic population in the initial must samples. The high microbial biodiversity within initial must samples was mostly due to environmental microorganisms derived from vineyard. In the freshly crushed grape must/juice from Australia, fungal communities were highly diverse and characterized by ubiquitous genera such as *Aureobasidium, Cladosporium, Saccharomyces,* and *Rhodotorula,* deriving from the vineyard ecosystem **(Liu et al., 2021)**. On the other hand, filamentous fungi were surprisingly missed in fresh grape juice from the white variety.

Palava on DRBC agar medium according to **Felšöciová, Mašková and Kačániová (2018)**, but the initial yeast diversity rapidly evolved in extremely stressful conditions, dominated by high sugar and low initial temperatures and the concentration of yeasts was 1 x 10^4 CFU.mL^{-1}.

In wine were not detected any filamentous fungi but 2 genera of yeasts were recorded. The entire microbial community was mostly characterized by *Saccharomyces cerevisiae* (95%), and *Candida utilis* (5%). **Felšöciová, Mašková and Kačániová (2018)** referred that at the end of the fermentation process only a few strains of yeasts survived (7.4 x 10^3 CFU.mL^{-1}) in wine sample from white variety Palava.

CONCLUSION

Four red grape varieties Alibernet, Dornfelder, Blue Frankish, Cabernet Sauvignon, and four white grape varieties Green Veltliner, Rheinriesling, Pinot Blanc, Sauvignon, from Small Carpathian wine growing region were analyzed by direct plating methods and must and wine by plate dilution method. In total, 410 isolates of filamentous fungi were identified by morphological analyses and 232 isolates of yeast by MALDI-TOF. From red grape varieties, 249 microbial isolates and 393 from white grape varieties were isolated, among which the filamentous fungi represented 64% of all isolates. The most abundant genera were *Penicillium, Hanseniaspora uvarum* and *Botrytis* from the total fungi accounted.

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The authors declare no conflict of interest.

Ethical Statement:

This article does not contain any studies that would require an ethical statement.

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