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MYCOBIOTA, ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT OF TOKAJ GRAPE VARIETIES

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ABSTRACT

Grape berries provide a natural habitat for diverse microorganisms, including potentially toxinogenic species. This study investigated the exogenous and endogenous mycocenosis of grapes, focusing on *Aspergillus* and *Penicillium* species and their toxinogenic potential, alongside the antioxidant profile (antioxidant activity and total polyphenol content) of four grape samples from the Tokaj wine-growing region in 2023. Exogenous and endogenous fungal communities were analyzed using plating methods, with surface disinfection for endogenous mycobiota. Micromycetes were identified morphologically, and yeasts were characterized using MALDI-TOF MS Biotyper. We isolated 458 fungal colonies across four genera (*Aspergillus, Penicillium, Rhizopus*, and *Trichoderma*) from exogenous mycocenosis, with *Penicillium* being the most frequent and abundant. From endogenous mycocenosis, 202 isolates across six genera (*Alternaria, Aspergillus, Cladosporium, Penicillium, Rhizopus*, and *Trichoderma*) were identified, with *Penicillium* dominating. Yeasts detected included *Pichia terricola, Aureobasidium pullulans, Hanseniaspora uvarum,* and *Pichia fermentans*. Toxinogenicity testing revealed that *Penicillium expansum* produced citrinin, while *Aspergillus clavatus* did not produce patulin under *in vitro* conditions. The Zéta grape variety showed the highest antioxidant activity (1.49 mg TEAC/g FM) and polyphenol content (3.15 mg GAE/g FM). These findings underscore the dual role of grapes as a source of valuable phytochemicals and a potential habitat for toxin-producing fungi, which could impact grape safety and quality. Future research should explore the relationship between grape microbiome composition and phytochemical profiles, as well as expand the study to other wine-growing regions to support sustainable grape production and food safety.

Keywords: microscopic filamentous fungi, antioxidant activity, polyphenols, Furmint, Lipovina, Yellow Muscat, Zéta

INTRODUCTION

At present, according to the legal classification, there are six viticultural areas in the Slovak Republic: Small Carpathians, South Slovakia, Central Slovakia, Nitra, East Slovakia, and Tokaj (**ÚKSÚP, 2023a**). Tokaj is one of the smallest vineyards in Slovakia, yet it is considered one of the most precious pearls among the wine-growing regions of the world due to its importance and history (**Hronský, 2017**). This region extends over the cadastral territory of the original vineyards of the Slanské Hills in the south, south-west, and south-east of Zemplín. Tokaj includes seven villages: Bara, Čerhov, Černochov, Malá Tŕňa, Slovenské Nové Mesto, Veľká Tŕňa, and Viničky, with a currently registered vineyard area of 89 hectares in Slovakia (**ÚKSÚP, 2023b**). The Tokaj region is distinguished by its unique features, which contribute significantly to its importance within the Slovak and international wine landscapes. Its climate is characterized by warm, mild, and dry summers, cool and wet winters, and long, dry autumns, which are essential for grape cultivation. Another unique feature is the regular occurrence of foggy autumn mornings, critical for the formation and development of the noble rot *Botrytis cinerea* on grapes (**Hronský, 2017**). The soil composition, which includes volcanic and clay sediments, enhances the terroir's ability to produce high-quality wines. This combination of climatic and soil conditions, along with traditional wine-making techniques, contributes to Tokaj's global reputation.

The surface of the grape berry represents an unstable habitat, with the presence of microorganisms varying significantly due to environmental factors, such as rapid temperature changes, humidity shifts, UV radiation, or insufficient water and nutrient availability. **Rogiers *et al.* (2022)** emphasized that the grapevine microcenosis is also influenced by agrotechnical and biological interventions made by growers. This habitat hosts yeasts, various bacterial species, and microscopic filamentous fungi, with yeasts being the most significant group (**Regecová *et al.*, 2022**). Commonly identified yeasts include *Aureobasidium pullulans*, *Hanseniaspora uvarum*, *Pichia*, *Metschnikowia*, *Candida*, *Saccharomyces*, *Rhodotorula*, *Cryptococcus*, *Kluyveromyces*, *Zygosaccharomyces*, *Dekkera*, and others. **Regecová *et al.* (2022)** stated that, regarding the identification of yeast populations on grape berries, the abundance and diversity of yeast species are influenced by factors such as grape variety, regional climate, vineyard elevation, disease occurrence, and grape damage. The ripening stage also plays a crucial role, as yeast populations are low on immature grapes but increase as the grapes ripen. Research from various wine-growing regions has demonstrated the influence of terroir on the composition of grape mycobiota, with significant differences observed between geographic areas. Studies conducted in Mediterranean regions, such as Italy and Spain, have highlighted a predominance of genera like *Botrytis, Aspergillus,* and *Penicillium* in vineyards with warmer climates, whereas cooler regions, including Central and Eastern Europe, report higher occurrences of *Cladosporium* and *Alternaria* species (**Pons *et al.*, 2021; Serra *et al.*, 2020**). The Tokaj region, due to its unique climatic and soil characteristics, is particularly conducive to the growth of *Botrytis cinerea,* which plays a key role in the production of noble rot wines. However, studies focusing specifically on the Tokaj grape mycobiota remain limited, necessitating further investigation to understand the microbial diversity in this region and its potential implications for wine quality and safety.

Understanding the microbial community (mycobiota) associated with grape berries is crucial, as it significantly impacts grape and wine quality, safety, and sensory characteristics. Microscopic filamentous fungi are also present on grape berries. **Kassemeyer (2017)** explained that some of these fungi are ubiquitous epiphytes that utilize sugars and amino acids from grape berries as nutrient sources, while others are pathogens that enzymatically inhibit ripening or destroy fruit tissue. Additionally, certain fungal species may produce flavor compounds or toxic metabolites. According to several studies, the most common micromycetes associated with grape rot belong to the genera *Penicillium*, *Aspergillus*, *Alternaria*, *Botrytis cinerea*, *Cladosporium*, and *Rhizopus* (**Felšöciová and Árvayová, 2022; Felšöciová *et al.*, 2018; Tournas and Katsoudas, 2005**). Other genera, such as *Trichoderma*, *Epicoccum*, *Fusarium*, and *Ulocladium*, may also occur. Grapes, like other fruits, can serve as a potential source of mycotoxins. However, **Welke (2019)** emphasized that visible disease symptoms on grapes do not necessarily indicate the presence of mycotoxins, and conversely, mycotoxins may be present even on visually healthy grapes. Several mycotoxins, including aflatoxins, ochratoxin A, citrinin, patulin, fumonisin B2, and roquefortin C, have been detected in grape berries (**Felšöciová *et al.*, 2015a, b; Felšöciová *et al.*, 2018**).

Numerous nutrients, including vitamins, mineral compounds, carbohydrates, dietary fiber, and phytochemicals, are present in grapes. **Xia *et al.* (2010)** noted that polyphenols are the most significant phytochemicals found in grapes, primarily responsible for their biological effects and health-promoting qualities. The primary types of phenolic compounds include anthocyanins, flavanols, flavonols, stilbenes (resveratrol), and phenolic acids. Grapes are also a rich source of flavonoids, particularly found in seeds and stems, such as (+)-catechins, (−)-epicatechin, and procyanidin polymers. In red grape varieties, anthocyanins serve as pigments in grape skins, while flavan-3-ols are more commonly found in white grape varieties.

The aim of our study was to monitor the exogenous and endogenous mycobiota of four grape varieties from the Tokaj wine region of Slovakia, focusing on the species spectrum of *Aspergillus* and *Penicillium* genera, their toxinogenicity, and the antioxidant profile of grapes.

MATERIAL AND METHODS

Sampling of grapes

Grape berry samples were obtained from a small winery in the Veľká Tŕňa area, part of the Tokaj wine-growing region. Four white grape varieties were selected: Furmint, Lipovina, Yellow Muscat, and Zéta. The samples were collected during the 2023 harvest in September and October, when the berries reached full ripeness. Each sample consisted of three bunches of grapes, which showed no visible signs of damage or mold growth. The berries were placed in sterile polyethylene packages and stored in the cold until analysis. Analyses were performed in the laboratories of the Department of Microbiology, Department of Technology and Quality of Plant Products, and the AgroBioTech Research Centre in Nitra.

Determination of exogenous mycocenosis of grape berries

To determine the exogenous mycocenosis of grape berries, we used the direct plating method **(Magnoli *et al*., 2003)**. We randomly selected 50 berries without damage and then plated to Petri dishes with a diameter of 140 mm (7–8 berries/dish) with Dichloran Rose Bengal Chloramphenicol Agar (DRBC; Merck, Germany). Petri plates were incubated at 25 °C for 5–7 days.

Determination of endogenous mycocenosis of grape berries

To determine the endogenous mycocenosis of grape berries, we used the direct plating method with surface disinfection **(Magnoli *et al*., 2003)**. Before placing the berries in Petri dishes, we sterilized the surface by rinsing with 1% chloramine solution for 120 seconds, rinsed 3 times with sterile distilled water (total volume 1 L) dried on sterile filter paper and placed into Petri dishes, as in the determination of exogenous mycocenosis. Cultivation took place at a temperature of 25 °C for 5–7 days.

Yeast identification

The identification of yeasts isolated from grape samples was performed using MALDI-TOF MS Biotyper (Daltonics, Bremen, Germany). Yeast was isolated from DRBC and inoculated onto Malt Extract Agar (MEA; Merck, Germany) **(Samson *et al.,* 2010)** and cultivated for 48 h at 25 °C. After cultivation, the yeast colonies were transferred into a 1.5 mL Eppendorf tube containing 300 µL of distilled water and mixed, 900 µL of absolute ethanol (Bruker Daltonik, Bremen, Germany) was added and the sample was centrifuged at maximum speed for 120 seconds. After centrifugation, the supernatant was removed and allowed the (v/v) (Sigma-Aldrich, USA) was added, mixed, added 50 µL of acetonitrile (Sigma-Aldrich, USA) and mixed again. Then the prepared sample was centrifuged at maximum speed for 120 seconds. In the final step, 1 µL of the newly formed supernatant was transferred to a MALDI - TOF plate and allowed to dry at laboratory temperature before overlaying with 1 µL of the matrix solution. The matrix was prepared by mixing 500 µL of acetonitrile, 25 µL of tri-fluoroacetic acid (Sigma-Aldrich, USA) and 475 µL of distilled water. In the next step, 250 µL of the prepared solvent was added to the Eppendorf flask with "HCCA matrix portioned". The Eppendorf was placed in a vortex and left stirred until the matrix crystals were completely dissolved. The plate thus prepared was placed in the ionization chamber of the mass spectrometer, where the protein spectra of the isolated sample were compared with the reference spectra in the MALDI - TOF MS Biotyper database. A score between 2.000 and 2.299 indicated a secure genus identification with probable species identification, a score between 1.700 and 1.999 suggestedprobable identification at the genus level, and a score below 1.700 was considered unreliable for identification.

Identification of micromycetes

During the identification of micromycetes, their macroscopic and microscopic features were evaluated according to the mycological guidance of **Pitt and Hocking (2009)**. Colony morphology, including color, texture, and size, as well as the production of conidia and spore structures, was examined. Microscopic features, such as conidiophores, conidia, and other reproductive structures, were observed. The focus was on species identification, particularly for the genera *Aspergillus* and *Penicillium*. *Aspergillus* strains were cultivated on MEA, Czapek Yeast Agar (CYA; Merck, Germany) **(Pitt and Hocking, 2009)**, *and* Czapek Yeast Extract with 20% Sucrose (CY20S, Merck, Germany) **(Pitt and Hocking, 2009)**. *Penicillium* strains were incubated on MEA, CYA, Creatine Sucrose Agar (CREA) **(Samson *et al.,* 2010)** and Yeast Extract Agar (YES; Merck, Germany) **(Samson *et al.,* 2010)**. Cultivation was carried out at 25 °C for 7 days in the dark. Identification of *Aspergillus* strains was according to **Klich (2002), Pitt and Hocking (2009)**, and *Penicillium* strains according to **Samson and Frisvad (2004)** and **Pitt and Hocking (2009)**.

Data analysis

The occurrence of microscopic filamentous fungi on/in the analyzed grape berries expressed in terms of relative density (RD) and isolation frequency (IF). RD (%) is defined as the percentage of isolates of the species or genus present in the analyzed sample **(Gautam *et al.*, 2009)**. The relative density was calculated using the formula:

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

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

IF (%) is defined as the percentage of samples in which the species or genus occurred at least once. These values were calculated according to **González *et al.* (1998)** as follows:

$$IF\ (\%) = (ns/N) \times 100$$

where ns – number of samples with a species or genus, and N – total number of samples.

Determination of toxinogenity of *Aspergillus* and *Penicillium* species by TLC method

Species of the genera *Aspergillus* and *Penicillium* were tested *in vitro* for their ability to produce mycotoxins by thin-layer chromatography (TLC) according to **Samson *et al.* (2002)** and modified by **Labuda and Tančinová (2006)**.

YES medium was used for screening of the extracellular mycotoxins patulin and citrinin, and CYA medium for screening of the intracellular mycotoxin roquefortin C. From the colonies (after 14 days of incubation), together with the medium, sections of approximately 5x5 mm was cut and placed in Eppendorf tubes with 500 µL of chloroform – methanol in the ratio of 2:1 (Reachem, Slovak Republic). The extraction of the sections was carried out on a Vortex Genie® 2 (MO BIO Laboratories, Inc., Carlsbad, CA, USA) for 3 min. The liquid phases of the extracts of the individual isolates were applied to the TLC plate (Alugram® SIL G, Macherey-Nagel, Germany) in a volume of 30 µL together with 10 µL of standards (Sigma, Germany) and allowed to evolve in TEF solvent (toluene:ethyl acetate:formic acid; 5:4:1, toluene - Mikrochem, Slovak Republic; ethyl acetate and formic acid - Slavus, Slovak Republic). After elution, the plate was air dried. Mycotoxins were identified by comparison with appropriate reference standards for mycotoxins.

Patulin was visible in daylight after spraying the chromatographic plate with 0.5% solution of methylbenzothiazolone hydrochloride (MBTH; Merck, Germany) in methanol followed by heating to 130 °C for 8 min as a yellow-orange spot. Roquefortine C was visualized in daylight after application of $Ce(SO_4)_2$ x 4 H_2O solution as an orange spot. Citrinin was detected under UV light at 365 nm and visible as a yellow-green spot with a tail.

Sample preparation for measurement of antioxidant activity (DPPH) and detection of total polyphenol content

The fresh grape berries were used for preparation of ethanolic extract and an amount of 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 measurement of antioxidant activity (DPPH) and detection of total polyphenol 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-difenyl-1-picrylhydrazyl (DPPH) according to the procedures described by **Sanchéz-Moréno *et al.* (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). 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) in fresh matter.

Total polyphenol content

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 of gallic acid equivalents per fresh matter.

Statistical analysis

All experiments were carried out in triplicate. The standard deviations for each replication were used for calculation of values of phytochemical and antioxidant properties of grapes. The experimental data were subjected to analysis of variance (Duncan's test) at the confidence level of 0.05 (SAS, 2009).

RESULTS AND DISCUSSION

Exogenous mycocenosis of analyzed grape berries

From 4 grape samples from exogenous mycocenosis we identified 4 genera of microscopic filamentous fungi: *Aspergillus, Penicillium, Rhizopus* and *Trichoderma* (Tab. 1). Approximately 1% of the isolated micromycetes did not form any fructifying structures necessary for their identification and were determined to be non-sporulating *Mycelia sterilia*. The total number of isolates of microscopic filamentous fungi was 458. The genus *Trichoderma* was the most abundantly represented genus, which was isolated 214 times from the samples (46.73% RD), followed by *Penicillium* (46.51% RD), which was detected in all 4 varieties. Among the representatives of the genus *Penicillium*, we isolated the species *P. expansum*, which had a relative density of 42.36% of all 458 isolates. The genus *Rhizopus* had the third highest relative density value of 5.68%, with 26 isolates. The smallest portion of exogenous colonization had the genus *Aspergillus* (0.22% RD), specifically the species *A. clavatus*.

Table 1 Numbers of isolates of microscopic filamentous fungi from exogenous grape mycocenosis, isolation frequency and relative density

Isolated genus/species	Grape varieties				Total number	IF	RD
	F	L	YM	Zéta		(%)	(%)
Aspergillus	-	-	1	-	**1**	**25**	**0.22**
A. clavatus	-	-	1	-	**1**	**25**	**0.22**
Mycelia sterilia	1	2	1	-	**4**	**75**	**0.87**
Penicillium	61	131	2	19	**213**	**100**	**46.51**
P. expansum	61	131	2	-	**194**	**75**	**42.36**
***P.* sp.**	-	-	-	19	**19**	**25**	**4.15**
Rhizopus	-	-	-	26	**26**	**25**	**5.68**
Trichoderma	98	116	-	-	**214**	**50**	**46.73**
Total	**160**	**249**	**4**	**45**	**458**		

Legend: F – Furmint, L – Lipovina, YM– Yellow Muscat, IF - Isolation frequency, RD - Relative density, *P*. sp. - *Penicillium* species

The highest number of 249 isolates was captured in the variety Lipovina (*Penicillium, Trichoderma* and *Mycelia sterilia*). The second most colonized variety by the same micromycetes with 160 isolates was the variety Furmint. This was followed by the variety Zéta (*Penicillium* and *Rhizopus*) with 45 isolates. The lowest number of isolates of 4 was recorded in the variety Yellow Muscat (*Aspergillus, Penicillium* and *Mycelia sterilia*).

The highest numbers of penicillium isolates in exogenous colonization were observed in the variety Lipovina (131). This was followed by Furmint (61). In the variety Zéta we isolated 19 isolates of *Penicillium* without identification of species, because the Petri dishes were overgrown with the genus *Rhizopus*. According to the frequency of occurrence, we detected the genus *Penicillium* in every variety studied, representing 100% frequency, with *Penicillium expansum* species dominating (75%). *Mycelia sterilia* had a frequency of occurrence of 75%, its presence in the samples of the varieties Furmint, Lipovina and Yellow Muscat. The genus *Trichoderma* was detected in the 2 varieties studied, Furmint and Lipovina, thus its frequency of occurrence was 50%. The genera *Aspergillus* and *Rhizopus* had the lowest frequency of occurrence of 25% in the samples. The genus *Rhizopus* was isolated only from the variety Zéta, and a representative of the genus *Aspergillus* was detected in the variety Yellow Muscat.

A similar mycological analysis was also carried out by **Felšöciová *et al.* (2015a)** in 2012, where they monitored the exogenous colonization of 3 grape varieties Furmint, Lipovina and Yellow Muscat from the Tokaj wine-growing region (village Viničky). In their study, nine genera of microscopic filamentous fungi were isolated, including *Alternaria, Aspergillus, Botrytis cinerea, Cladosporium, Mucor, Penicillium, Phoma, Rhizopus*, and *Trichoderma*. The highest frequency of occurrence of 100% was recorded for *Aspergillus, Botrytis* and *Penicillium* genera. This was followed by the genera *Alternaria, Cladosporium* and *Rhizopus* with 67% IF. Six species of microscopic filamentous fungi from the genus *Penicillium* were identified, with *P. expansum* and *P. chrysogenum* being the predominant species (100%). Compared with our results, the genera *Aspergillus, Penicillium, Rhizopus* and *Trichoderma* were also isolated from our samples. The genus *Penicillium* (100%) was also the most abundant genus on the surface of the grape samples, with *P. expansum* being the dominant species as well, with a relative density of 42.36%. In contrast, our results do not show exogenous contamination by the genera *Alternaria, Cladosporium, Botrytis cinerea, Mucor* and *Phoma*. Colonization of grape berries by the genus *Aspergillus* was very low in our case, its relative density was 0.22%, the same in the results of their study (1.1%). The genus *Penicillium*, particularly *P. expansum*, is known for its ability to produce patulin, a mycotoxin that poses significant health risks if present in food products, including grape derivatives (**Frisvad *et al.*, 2004; Zhang *et al.*, 2021**). While our study confirmed the dominance of *Penicillium* (100%), it is crucial to highlight the potential risk associated with its presence, emphasizing the need for regular monitoring during grape processing to ensure food safety. Additionally, **Kunová *et al.* (2018)** investigated the presence of microscopic filamentous fungi on grape berries from the Small Carpathian wine-growing region. Their analysis of 13 grape samples (9 white and 4 blue varieties) identified eight genera of micromycetes, including *Alternaria, Aspergillus, Botrytis, Cladosporium, Penicillium, Phoma, Rhizopus,* and *Trichoderma*. Notably, the genera *Alternaria* and *Penicillium* displayed a 100% frequency of occurrence. Similarly, higher values were recorded for the genera *Aspergillus* (77%), *Cladosporium* (77%), and *Botrytis* (54%). Four of these genera —*Aspergillus, Penicillium, Rhizopus,* and *Trichoderma* were also recorded in our samples, with *Penicillium* achieving a 100% frequency of occurrence. In contrast to *Aspergillus*, which is more commonly associated with warmer and tropical climates (**Serra *et al.*, 2006**), the dominance of *Penicillium* in temperate regions, such as Tokaj and the Small Carpathians, aligns with our findings. This supports the hypothesis that climate conditions significantly influence the composition of the grape mycobiota (**Rybárik *et al.*, 2014; Kunová *et al.*, 2018**). The minimal presence of *Aspergillus* in our study further confirms this regional pattern. **Felšöciová *et al.* (2017**) exogenously tested 8 grape samples between 2011 and 2013 (7 white and 1 blue variety) from the South Slovak wine-growing region. The frequency of occurrence 100% were colonies of the genus *Penicillium* and 75% of the genus *Aspergillus*. The main species of the genus *Penicillium* in the samples were *P. expansum* with a relative density of 37.5%, followed by *P. citrinum, P. chrysogenum* and *P. crustosum*. We came to similar conclusions for the genus *Penicillium*. From the *Penicillium* we isolated the main species *P. expansum*, which had a relative density of 42.36%. However, the genus *Aspergillus* in our samples was not dominant, (25% IF and 0.22% RD). Moreover, **Serra *et al.* (2006)** reported that the genus *Aspergillus* appears more frequently in warmer and tropical climates, while the genus *Penicillium* is more associated with temperate and cooler regions. These climatic influences are critical not only for understanding regional variations in mycobiota but also for assessing potential risks to grape safety and wine

production. As *Penicillium* and *Aspergillus* are known producers of mycotoxins like patulin and ochratoxin A, their presence could indirectly affect the sensory and safety characteristics of wines made from contaminated grapes (**Wei *et al.*, 2022**). Similarly, **Rybárik *et al.* (2014)** monitored the exogenous colonization of 10 grape samples from the Central Slovakian wine-growing region in 2011 and 2012. In total, they isolated 17 genera of microscopic filamentous fungi, with two genera, *Aspergillus* and *Penicillium*, having 100% frequency of occurrence. Consistent with these findings, *Penicillium* was also predominant in our analysis, whereas the genus *Aspergillus* was less dominant. **Wei *et al.* (2022)** reported that the genera *Penicillium* and *Aspergillus* have received increased attention in many studies as they are considered as pathogens directly responsible for grape rot. In addition, these genera are indirectly involved in spoilage by producing mycotoxins which can greatly affect the safety characteristics or sensory quality of the wine. However, these micromycetes are unable to grow and survive in wines and their impact on wine quality is due to grape deterioration.

Endogenous mycocenosis of analyzed grape berries

From 4 sterilised samples of grapes of the varieties Furmint, Lipovina, Yellow Muscat and Zéta we identified 6 genera of microscopic filamentous fungi: *Alternaria, Aspergillus, Cladosporium, Penicillium, Rhizopus* and *Trichoderma* (Tab. 2). Approximately 2% of the isolated fungi, as in the assessment of exogenous mycocenosis, did not produce any fructifying structures and were evaluated as non-sporulating *Mycelia sterilia*. The total number of isolates of microscopic filamentous fungi from the varieties studied was 202. The most represented genus was *Penicillium*, which was again detected in all 4 varieties with the highest relative density of 67.82%, with a number of isolates of 137. Like the exogenous colonization, from the representatives of the genus *Penicillium*, we isolated the species *P. expansum*, which had a relative density value of 65.84% from all 202 isolates. It was most abundant in the Furmint variety, where it accounted for 68 isolates. The genus *Rhizopus* had the second highest overall relative density of 10.89%, with a total number of isolates of 22, followed by the genus *Alternaria*, which had a relative density of 9.42%. The genus *Trichoderma* (4.95%) was next in order with a lower relative density. The genera *Aspergillus*, namely *A. clavatus* and *A. niger* complex species, and *Cladosporium*, with a relative density of 2.48%, had the lowest contribution to endogenous colonization.

Table 2 Numbers of isolates of microscopic filamentous fungi from endogenous grape mycocenosis, isolation frequency and relative density

Isolated genus/species	Grape varieties				Total number	IF	RD
	F	L	YM	Zéta		(%)	(%)
Alternaria	2	2	11	4	19	100	9.41
Aspergillus	1	-	3	1	5	75	2.48
A. clavatus	1	-	2	1	4	75	1.98
***A. niger* complex**	-	-	1	-	1	25	0.5
Cladosporium	-	-	5	-	5	25	2.48
Mycelia sterilia	2	1	1	-	4	75	1.98
Penicillium	68	38	11	20	137	100	67.82
P. expansum	68	38	11	16	133	100	65.84
***P.* sp.**	-	-	-	4	4	25	1.98
Rhizopus	-	-	4	18	22	50	10.89
Trichoderma	10	-	-	-	10	25	4.95
Total	**83**	**41**	**35**	**43**	**202**		

Legend: F – Furmint, L – Lipovina, YM – Yellow Muscat, IF - Isolation frequency, RD - Relative density, *P.* sp. - *Penicillium* species

According to the frequency of occurrence in the samples, we detected the genera *Alternaria* and *Penicillium* in each variety studied (Furmint, Lipovina, Yellow Muscat and Zéta), which represents 100 % of frequency (Tab. 2). The genera *Aspergillus* and *Mycelia sterilia* had a frequency of occurrence of 75%, their presence varied among the varieties sampled. The lowest frequency of occurrence of 25 % in the samples was in the genus *Cladosporium*, which was detected in Yellow Muscat.

The highest number of 83 isolates was recorded in the variety Furmint (*Alternaria, Aspergillus, Penicillium, Trichoderma* and *Mycelia sterilia*). The second most colonized variety by micromycetes with 43 isolates was Zéta (*Alternaria, Aspergillus, Penicillium* and *Rhizopus*). This was followed by the variety Lipovina with 41 isolates (*Alternaria, Penicillium* and *Mycelia sterilia*). The lowest number of isolates was 35, but with the highest number of genera in the variety Yellow Muscat (*Alternaria, Aspergillus, Cladosporium, Penicillium, Rhizopus* and *Mycelia sterilia*).

Felšöciová *et al.* (2013) stated that their study reported the capture of microscopic filamentous fungi from endogenous mycocenosis in grape samples of Furmint, Lipovina, and Yellow Muscat from the Tokaj wine-growing region, which aligns with our study. They isolated 12 genera of microscopic filamentous fungi from the tested samples. The most frequently occurring genera were *Alternaria, Aspergillus, Botrytis, Penicillium* and *Trichoderma*. They identified 8 species of *Penicillium*, the most abundant was *P. expansum*, which had a relative density of 24% of all penicillium species. The genera *Alternaria* (100%), *Penicillium* (100%) and *Aspergillus* (75%) were also the most abundant in our samples. The dominant species of the genus *Penicillium*, in our study, was also *P. expansum* but with a higher relative density of 65.84%. However, our results do not demonstrate endogenous colonization by the genus *Botrytis*. **Rybárik *et al.* (2014)** noted in their study from 2011 and 2012 that they investigated not only exogenous but also endogenous colonization in 10 grape samples from the Central Slovakian wine-growing region. In total, they isolated 15 genera of microscopic filamentous fungi, with *Alternaria* being the most frequently occurring genus (100% IF), followed by *Cladosporium* and *Fusarium* with 90% IF. The genus *Alternaria* also recorded the highest number of isolates with a relative density of 48.68%. The genera *Penicillium* (20.64%) and *Cladosporium* (13.61%) also showed high values. The four genera *Fusarium, Botrytis, Epicoccum* and *Rhizopus* had relative densities between 1 - 5%, less than 1% was recorded for the genera *Trichoderma* (0.91%), *Aspergillus* (0.73%), *Mucor* (0.09%) and *Phoma* (0.09%). Compared to our results, for the same most frequently occurring genus *Alternaria*, with 100% frequency of occurrence, we observed higher relative densities of isolates for the genera *Penicillium* (67.82%), *Rhizopus* (10.89%), *Trichoderma* (4.95%) and *Aspergillus* (2.48%), but the relative densities were lower for the genera *Alternaria* (9.41%) and *Cladosporium* (2.48%). In contrast to their findings, the genera *Fusarium, Botrytis, Epicoccum, Mucor* and *Phoma* were not observed in our analyzed samples.

The genera of microscopic filamentous fungi identified by us, *Alternaria, Aspergillus, Cladosporium, Penicillium, Rhizopus* and *Trichoderma*, have been isolated in other countries and reported in various studies. **Varga *et al.* (2007)** reported that they collected 35 grape samples from 20 different Hungarian vineyards in 2004 and identified the genera *Penicillium, Botrytis, Alternaria, Trichoderma* and *Cladosporium*. They also collected 22 grape samples from 5 vineyards in the Znojmo wine-growing region (Czech Republic) and isolated the genera *Alternaria, Cladosporium, Penicillium, Rhizopus, Epicoccum* and *Aspergillus*. Similarly, **Serra *et al.* (2005)** emphasized that *Alternaria, Aspergillus, Botrytis, Cladosporium* and *Penicillium* were the most frequently occurring genera in grape samples from Portuguese vineyards.

When comparing these findings with several studies, we observed that a similar spectrum of genera of microscopic filamentous fungi from exogenous and endogenous colonization of grape berries has been consistently reported. From our point of view, an interesting finding was that the genus *Botrytis* was not present in our analyzed samples, in contrast to other studies. As **Fournier *et al.* (2013)** explained, *Botrytis cinerea* can cause two distinct types of infections on grapes, namely botrytis rot (grey mould), which has a highly damaging effect, and noble

rot, also known as "botrytization," which occurs under favorable climatic conditions. They further elaborated that grey rot typically develops during periods of high air humidity (above 90%) and prolonged wet weather, while noble rot requires cooler, misty nights and sunny, dry autumn days. **Dankó *et al.* (2021)** highlighted that noble rot development is a favorable process, resulting in shriveled, chocolate-brown berries enriched with aromatic compounds, which are essential for botrytized sweet wines. Considering these aspects, we assumed that *Botrytis cinerea* (noble form), characteristic of the Tokaj region and involved in forming the "cibeba," would be part of the mycocenosis of our samples, as favorable autumn climatic conditions might have supported its development. However, we believe that its absence in our study may be related to the earlier sampling period, which took place during grape ripening (September to early October).

Yeast of analyzed grape berries

Each grape variety was characterised by a high yeast content, but because of the rich presence of microscopic filamentous fungi, it was not possible to isolate them as pure cultures. The identification of yeasts was based on inter-comparison of mass spectra in MALDI - TOF MS Biotyper. In total, we identified 4 yeasts at species level.

From the exogenous mycocenosis of the berries analysed, we isolated and identified only one yeast species, namely *Pichia terricola*, which was detected in the variety Lipovina.

The yeast community within endogenous mycocenosis was slightly more diverse compared to exogenous colonization. Using MALDI - TOF MS Biotyper we identified 4 yeast species, namely *Aureobasidium pullulans, Hanseniaspora uvarum, Pichia fermentans* and *Pichia terricola*. The richest species composition of yeasts was recorded in the variety Yellow Muscat. In this variety all the above species have been identified. In the Lipovina sample we detected 2 yeast species, namely *Pichia terricola* and *Hanseniaspora uvarum*, which represented the second highest total number of yeasts detected. In the Furmint and Zéta cultivars, again, no yeast species were identified.

The yeast *Aureobasidium pullulans* was also isolated by **Serra *et al.* (2005)** in Portuguese grape varieties. Similarly, **Sage *et al.* (2002)** reported in their study the capture of this yeast from vineyards in France. *Aureobasidium pullulans* has also been isolated from Slovak vineyards. For instance, **Kántor *et al.* (2017)** microbiologically analysed several grape varieties harvested in the Central Slovakian, Tokaj and Nitra wine-growing regions of Slovakia. They identified the presence of different yeast species, including *Aureobasidium pullulans* and *Hanseniaspora uvarum*, by means of the MALDI - TOF MS, as we did. In addition, **Kačaniová *et al.* (2019)** identified the yeast *Hanseniaspora uvarum* from the Small Carpathian wine-growing region. Similarly, **Kraková *et al.* (2012)**, while monitoring yeast diversity in grape and grape must samples from the Small Carpathian wine-growing region, confirmed the presence of *Hanseniaspora uvarum* and *Pichia fermentans* yeasts. According to **Wei *et al.* (2022)**, the species abundance of microorganisms on grape berries, including yeasts, can vary significantly. This variation is influenced by factors such as climatic conditions (e.g., rain, humidity, and temperature), vineyard geography (e.g., altitude, latitude, and longitude), grape variety, ripening stage, berry health, and the application of pesticides and fertilizers.

Toxinogenity of aspergilli and pencilia from exogenous and endogenous colonization of grapes

We tested 19 isolates of *P. expansum* for their ability to produce the mycotoxins patulin, citrinin and roquefortine C and 2 isolates of *A. clavatus* for their ability to produce patulin under *in vitro* conditions using TLC. From the exogenous colonization of grapes, we tested 5 isolates of *P. expansum* and 1 isolate of *A. clavatus*. All 5 isolates of *P. expansum* tested were able to produce citrinin, 4 isolates patulin and only 1 isolate produced roquefortin C. The *A. clavatus* isolate was not shown to be productive for patulin.

From the endogenous colonization of grapes, 14 isolates of *P. expansum* and 1 isolate of *A. clavatus* were tested. All 14 *P. expansum* isolates tested were able to produce citrinin, 11 isolates patulin and no isolate produced roquefortin C. The production of patulin, again by *A. clavatus* species, was not confirmed by TLC. These results are significant as they provide information about the potential mycotoxin risks that may be present in grapes and wine, while also allowing for a better understanding of the behavior of these species in the context of different stages of grape colonization. The results of testing isolates from exogenous and endogenous grape mycocenosis under *in vitro* conditions by thin-layer chromatography are shown in Table 3.

Table 3 Toxinogenity of aspergilli and pencilia from exogenous and endogenous colonization of grape berries confirmed by TLC

Tested species	citrinin	patulín	roquefortíne C
Toxinogenity from exogenous colonization of grape berries			
Aspergillus clavatus	-	0* / 1**	-
Penicillium expansum	5 / 5	4 / 5	1 / 5
Toxinogenity from endogenous colonization of grape berries			
Aspergillus clavatus	-	0 / 1	-
Penicillium expansum	14 / 14	11 / 14	0 / 14

Legend: * number of production isolates; ** total number of isolates tested

Felšöciová *et al.* (2015a) in their 2012 study on species of microscopic filamentous fungi with potential toxinogenic properties, analyzed 52 isolates obtained from grape berries of Furmint, Lipovina, and Yellow Muscat varieties originating from the Tokaj wine-growing region. They tested 18 strains of *P. expansum* for their ability to produce mycotoxins. Of the 18 *P. expansum* strains tested, 15 were able to produce patulin, 13 citrinin and all 18 isolates produced roquefortine C. Similarly, our tested strains were also productive for patulin, citrinin and roquefortin C, but the production of roquefortin C was lower in our strains. In a related study, **Felšöciová *et al.* (2015b)** evaluated the mycotoxicity of 5 isolates of *P. expansum* collected from grapes originating from the Small Carpathian wine-growing region during 2011–2013. Among these isolates, all 5 produced roquefortine C, 3 produced patulin, and 2 produced citrinin. Patulin is a toxin produced by several species of the genera *Aspergillus, Penicillium,* and *Paecilomyces* **(White *et al.*, 2006)**, especially by *P. expansum*. It is the most well-known toxin associated with fresh fruit and is considered more of a postharvest problem. It is primarily prevalent in apples and apple products, also in grapes, pears or oranges. Its occurrence can be attributed to the physicochemical properties of the fruit, such as its water activity and pH, which promote the growth of *P. expansum* **(Mahato *et al.*, 2021)**. Processes such as clarification, filtration and enzymatic treatment in the processing of fruit juices and fermentation in wine production significantly reduce the patulin content. Also washing, sorting and removal of damaged parts from fruit can reduce its content **(Mahato *et al.*, 2021)**.

Antioxidant activity and total polyphenol content

Antioxidant activity of grape varieties ranged from 0.77 to 1.49 mg TEAC/g FM by the DPPH method (Tab. 4). The highest antioxidant activity was detected in Zéta followed by Yellow Muscat variety. The observed differences in antioxidant activity between these varieties may be attributed to various factors such as grape variety, climate conditions, and agronomic practices (**Bajčan *et al.*, 2011**). Using the DPPH method, **Soyollkham *et al.* (2011)** determined that the antioxidant activity of Grüner Veltliner grape ranged from 0.29 to 0.35 mmol GAE/L (gallic acid equivalent). Additionally, they verified that red wines have 5-8 times more antioxidant activity than white wines. In study of **Fikselová *et al*. (2010)** fourteen quality wines from Tokaj area of Slovakia were analysed for their antioxidant and antimicrobial activity. All Tokaj wines showed very good antiradical effect (against DPPH radical), more than 50%, Tokaj essence (75.72%) and Tokaj 6 puttony (72.7-78%) showed the best antiradical activity. Antioxidant effect of wines expressed in % inhibition ranged from 57.61 to 78.00. The year of grape cultivation and botrytization of grape showed important influence on antioxidant status of wines. It is also worth considering that the grape's microbial community may interact with these antioxidants, either by producing metabolites that influence antioxidant levels or by protecting the grape from oxidative stress, which may further affect the overall phytochemical profile (**Barata *et al.*, 2012**).

Total polyphenols in analyzed grape varieties ranged from 1.11 to 3.15 mg GAE/g FM with the highest content in Zéta variety (Tab. 4). According to **Liang *et al.* (2014)**, the anthocyanins in grape skin are responsible for the greater total polyphenol content of colored grapes compared to green-yellow grapes. A darker grape's overall polyphenolic content is correlated with its color. This highlights that the coloration and polyphenolic richness of grapes can be influenced by both genetic factors and environmental conditions, such as sunlight exposure and temperature, which in turn may also interact with the microbiome of the grapevine. Polyphenols, secondary plant metabolites, consist of one or more aromatic rings substituted with hydroxyl groups and are predominantly located in the skin and seeds of grapes. During the vinification process, 70–120 kg of grape pomace is generated per ton of grapes on a dry matter basis (**Kulichová *et al*., 2018**). Grape pomace, a by-product of winemaking, is increasingly utilized in the food and feed industries due to its high polyphenol content. Research by **Gálik *et al.* (2018**) and **Juráček *et al*. (2018**) demonstrated that incorporating grape pomace into animal diets can improve both productivity and health. This suggests that polyphenols in grape by-products can have broader applications in food and health industries, especially considering their antioxidant and antimicrobial properties. The polyphenol content in grapes is influenced by the ripening stage and agro-ecological conditions. **Král *et al.* (2018)** reported a significant reduction in total polyphenols during ripening, especially in the Welschriesling variety. It is also

important to note that environmental stressors, such as drought or pest infestation, could impact both the polyphenol concentration and the microbial communities present in the vineyard. The presence of certain microorganisms, such as fungi, may influence the biosynthesis of polyphenols, creating a complex interaction between the plant and its microbial associates (**Chitarrini *et al*., 2017**). In the study conducted by **Bajčan *et al.* (2018)**, the highest total phenolic and flavonoid content was observed in Yellow Muscat Tokaj wine (525.6 ± 43.4 mg GAE/L). Furthermore, these authors confirmed a very strong linear correlation between antiradical activity and total polyphenol content (r = 0.867).

Table 4 Antioxidant activity and total polyphenol content of analyzed grape varieties

Sample	DPPH method [mg TEAC/g FM]	Total polyphenols [mg GAE/g FM]
Furmint	0.77±0.01^{d}	1.11±0.02^{d}
Lipovina	0.85±0.01^{c}	1.34±0.03^{c}
Yellow Muscat	1.07±0.02^{b}	1.34±0.06^{b}
Zéta	1.49±0.02^{a}	3.15±0.08^{a}

Legend: DPPH - 2,2-difenyl-1-picrylhydrazyl; TEAC – Trolox equivalent antioxidant capacity; GAE – gallic acid equivalent; FM – fresh matter; mean ± standard deviation; different letters in column denote mean values that statistically differ one from another

The findings on grape microbiota, mycotoxins, and antioxidants offer valuable applications for viticulture, food safety, and the wine industry. Understanding the grape microbiota helps in managing spoilage and contamination risks, while identifying mycotoxin-producing fungi can guide the development of monitoring systems to ensure wine safety. The antioxidant activity data can be used to select grape varieties with higher antioxidant potential, improving wine quality and nutritional value. These insights contribute to safer production practices, higher-quality wines, and the utilization of grape by-products in various industries.

CONCLUSION

Experienced winemakers often emphasize that good wine starts in the vineyard, where every aspect—from the winegrower's practices, grape variety, and geological conditions to climate and soil—affects the final product. However, beyond these factors, the quality of grapes is also significantly influenced by the microorganisms that inhabit them. Some microbes promote grape health, while others can cause diseases or spoilage. Grapes, being rich in sugars and nutrients and having ideal conditions for microbial growth, are particularly susceptible to contamination by microscopic filamentous fungi, including toxinogenic species, which can result in the presence of harmful mycotoxins. Our study revealed the presence of potentially toxigenic genera such as *Aspergillus* and *Penicillium*, with *Penicillium expansum* being the dominant species. In both exogenous and endogenous mycocenosis, the genus *Penicillium* represented the most frequent and abundant species, with 100% occurrence and high relative density values (46.51% from exogenous and 67.82% from endogenous mycocenosis). *Aspergillus* species were found to a much lesser extent. These findings underline the importance of monitoring grape mycobiota, especially for toxigenic fungi, to ensure the safety and quality of wine grapes and, ultimately, wine. The presence of mycotoxins in grapes can pose health risks. To reduce these risks, comprehensive vineyard management practices, including early pathogen detection, proper identification of diseases, targeted phytosanitary measures, and the careful sorting of damaged and rotten berries, are crucial. Such practices can significantly minimize the growth of pathogenic fungi and the production of mycotoxins. In addition to the microbial risks, our research also highlighted the significant antioxidant activity and polyphenol content in the Zéta grape variety, which showed the highest antioxidant activity compared to other varieties analyzed. The high polyphenol content is beneficial not only for the health of consumers but also for improving the sensory and technical characteristics of wines.

Future research could explore expanding the study to a broader range of grape varieties, vintages, and geographical regions to further understand the interplay between grape microbiota, phytochemicals, and wine quality. By applying these insights, wine producers can improve the safety, quality, and sustainability of wine production, enhancing the value of wines.

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