Yeasts associated with the spontaneously fermented grape musts obtained from cool climate white grape varieties

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Summary

The study dealt with quantitative and qualitative analysis of yeast microbiota found during spontaneous fermentation of grape musts from the white grape varieties Hibernal, Bianca and Seyval Blanc. Yeast strains were differentiated by random amplification of polymorphic DNA and pre-identified by restriction fragment length polymorphism of the 5.8S-ITS rRNA gene region. Final identification was obtained by 5.8S-ITS rRNA gene region sequencing. *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Zygoascus meyerae*, *Wickerhamomyces anomalus* and *Kluyveromyces lactis* strains were identified in all fermented musts. Higher yeast counts were determined in fresh musts from 2012 season compared to 2013, which directly affected their quantitative composition during the process. Musts obtained from the grapes from the Spotkaniówka vineyard were characterized by a higher microbial content compared to those obtained from the Srebrna Góra vineyard. It could be related to the microclimatic conditions in the vineyard. The grape varieties currently grown in Poland are characteristic for the cool climate region. Detailed studies on the microbiota of grapes and grape must allow for identification of yeast strains characteristic for a specific terroir and defining the 'identity' of a regional wine.

Keywords

variety Bianca; variety Hibernal; variety Seyval Blanc; spontaneous fermentation; yeast ecology; cool climate

One of the most important technological advances in viticulture was the inoculation of grape juice with *Saccharomyces cerevisiae* strains, which enabled to control the fermentation process. *S. cerevisiae* outcompete non-*Saccharomyces* species due to specific characteristics, such as higher fermentative power, alcohol tolerance, and resistance to low pH, scarce oxygen availability or depletion of certain nutrients. However, numerous studies showed that non-*Saccharomyces* yeasts in grape must are widespread and occur at different stages of fermentation simultaneously with *S. cerevisiae* strains [1].

During spontaneous fermentation, a succession of the most important yeast groups is observed. It involves occurrence of individual representatives of the genera *Hanseniaspora* (anamorph *Kloeckera*), *Metschnikowia*, *Candida* and *Saccharomyces*. To a large extent, it contributes to shaping the desired sensory characteristics of wine. On the other hand, presence of native non-*Saccharomyces* strains during fermentation could results in the appearance of undesirable characteristics, including high levels of acetic acid, ethyl acetate, ethanol and/or acetoin. Moreover, most of these strains are characterized by a reduced fermentation rate and high sensitivity to SO_2 [2]. Despite the unfavourable characteristics, several studies were carried out regarding the presence and activity of non-*Saccharomyces* yeasts in the grape must. A thorough analysis of their fermentation properties or impact on the complexity of the final aroma of wine has updated previous views [3].

In the early stages of fermentation, the content of non-*Saccharomyces* yeasts ranges from 10^3–10^5 CFU·ml^{-1} to 10^6–10^7 CFU·ml^{-1} [4]. Research indicates their great diversity during the first 24–72 h of the process. The most common

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include *Candida*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Torulaspora* and *Saccharomyces* species. *Dekkera*, *Schizosaccharomyces* and *Zygosaccharomyces* species are much less frequently identified [5].

In most countries, wine production is based on the use of commercial yeast strains as a starter culture. However, research shows that the vineyard could be also a source of native cultures with some favourable oenological properties [6]. To this date, many experiments have been conducted regarding the composition of yeast microbiota and species succession on the surface of grapes and during the fermentation of grape musts. However, there is little information on yeasts associated with grapevine varieties cultivated in cool regions. By the decision of the Council of the European Union of 20 December 2005, the territory of Poland belongs to zone A (the coldest) of vine-growing zones in Europe. This zone, referred to as “cool climate”, is usually characterized by an average temperature of about 15 °C in the month preceding the harvest. Due to climatic and soil conditions, the obtained grapes are characterized by a lower content of sugars (usually 17–23 %) and thus a low level of alcohol, higher acidity and higher content of polyphenolic compounds [7]. It is known that the process of must fermentation is influenced by soil, climate, exposition, but also by the fruit variety. For this reason, examining its impact on the quantitative and qualitative composition of microorganisms involved in the spontaneous fermentation process seems to be an important issue. The obtained knowledge can help the winemakers to better manage or control this process and to promote development of winemaking in cooler regions, such as Poland.

The aim of the study was to characterize the yeast microbiota found during spontaneous fermentation of grape musts obtained from white grape varieties ‘Hibernal’, ‘Bianca’ and ‘Seyval Blanc’, grown in cool climate areas.

MATERIALS AND METHODS

Grapes and spontaneous must fermentation

Grapes of three grapevine varieties (‘Hibernal’, ‘Bianca’ and ‘Seyval Blanc’) from two vineyards located in southern Poland (Srebrna Góra – 50°2’N, 19°50’E and Spotkaniówka – 49°53’ N, 21°52’E) during two consecutive vintages (2012 and 2013) were used in the study (Tab. 1). The grapes were harvested at full maturity. The ‘Hibernal’, ‘Bianca’ and ‘Seyval Blanc’ grape varieties are used for the production of white wine. These varieties are very popular in Poland. They are relatively resistant to frost and diseases.

Tab. 1. Grape varieties used in the study and dates of harvest.

Grape variety	Vineyard	
	Srebrna Góra	Spotkaniówka
Hibernal	2.10.2012 8.10.2013	27.09.2012 28.09.2013
Bianca		27.09.2012 28.09.2013
Seyval Blanc	2.10.2012 8.10.2013	

Ten bunches of mature grapes were gathered from several grapevines within a sub-area of each vineyard (100 m^2). Then, berries were randomly selected (500 g), placed in sterile 500 ml flasks and pressed until juice covered the fruits. The flasks were closed with airlocks filled with glycerol. Fermentation was carried out for 28 days at a temperature of 20 °C in triplicate.

Physicochemical characteristics of grape musts

The pH, total acidity and sugar content of fresh musts were determined in accordance with the official International Organisation of Vine and Wine (OIV) methodology [8]. Determination of sugars was carried out using NEXERA XR apparatus with an RF-20A refractometric detector (Shimadzu, Kyoto, Japan). The separation was carried out on an Asahipak NH2P-50 250 mm × 4.6 mm Shodex column (Showa Denko Europe, Munich, Germany), thermostated at 30 °C. The mobile phase was acetonitrile (70 %), and the isocratic elution program (0.8 ml·min^{-1}) lasted 16 min. Other analyses were performed in accordance with the official OIV methodology [8].

Yeasts enumeration and isolation

One millilitre samples of the fresh and fermenting musts (the 1st, 2nd, 3rd, 4th, 6th, 9th, 13th, 18th, 24th, and 28th day of fermentation) were withdrawn under sterile conditions. Serial decimal dilutions were prepared from the samples taken and inoculated in six replicates on Petri dishes with Wallerstein Laboratory agar (WL Agar; Biocorp, Warszawa, Poland). To avoid bacterial growth, 100 mg·l^{-1} of chloramphenicol was added to the media. The media were incubated at 28 °C for 5 days, which was followed by a macro- and microscopic evaluation of the grown colonies and the determination of their count. Colonies with different morphologies (size, shape, colour) were randomly selected for identification and streaked on Sabouraud glucose with Chloramphenicol LAB-AGAR (Biocorp) to obtain pure cultures. *Hanseniaspora* strains were identified based on their morphological characteristics, as assessed macroscopically and microscopically.

Molecular analysis

The analyses were carried out in accordance with the methodology described by Cioch-Skoneczny et al. [9]. Isolates were typed by random amplification of polymorphic DNA – polymerase chain reaction (RAPD-PCR) in order to characterize the identical strains (which should have the same RAPD patterns) and to reduce the number of samples taken for further analysis. Cultures distinguished by different RAPD patterns were identified by 5.8S-ITS rRNA gene region sequencing.

5.8S-ITS rRNA gene region sequencing

The amplified product of the rRNA gene was purified using Clean up AX (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions and submitted for sequencing to Macrogen (Amsterdam, Netherlands). Species identification was achieved by comparing processed sequences with those available in the GenBank database (National Center for Biotechnology Information, Bethesda, Maryland, USA) using the basic local alignment search tool (BLAST). Percent homology scores were generated to identify yeast isolates. Sequences were deposited in the GenBank database with the following accession numbers: MG970696 (*Zygoascus meyerae)*, MG971249, MG971250, MG971253, MG971257 and MG971260 (*Metschnikowia pulcherrima*), MG971252 and MG971266 (*Hanseniaspora uvarum*), MG971257 (*Kluyveromces lactis*), MH020215 (*Saccharomyces cerevisiae*) and MG971261 (*Wickerhamomyces anomalus*).

Statistical analysis

Results presented in the paper were the means of three independent repetitions with determination of the standard deviation. The data were analysed by variance analysis (ANOVA) to establish the significance of tested parameters. Statistically significant differences between the means were verified by Duncan's test using Statistica 10 software (StatSoft, Tulsa, Oklahoma, USA).

RESULTS AND DISCUSSION

Yeast population kinetics

In the freshly pressed musts, the numbers of yeasts differed significantly between the vineyards and seasons. Fruits harvested in 2012 were characterized by a much better chemical composition (higher sugars content, lower total acidity), which influenced the amount of yeasts present in the analysed grape juices (Fig. 1, Fig. 2). In musts obtained in 2012 from Seyval Blanc grapes (Srebrna Góra vineyard) and Bianca grapes (Spotkaniówka vineyard), the content of yeasts was similar (9.5×10^6 CFU·ml^{-1} and 1.9×10^6 CFU·ml^{-1}, respectively). Slightly lower contents were observed in freshly pressed Hibernal grape juice (Fig. 1, Fig. 2). In 2013, a significantly lower content of yeasts was found in the musts from grapes from the Srebrna Góra vineyard (6×10^2 CFU·ml^{-1}). In musts from the Spotkaniówka vineyard, it reached a value higher by one logarithmic order (3.6×10^3 CFU·ml^{-1}) (Fig. 1, Fig. 2).

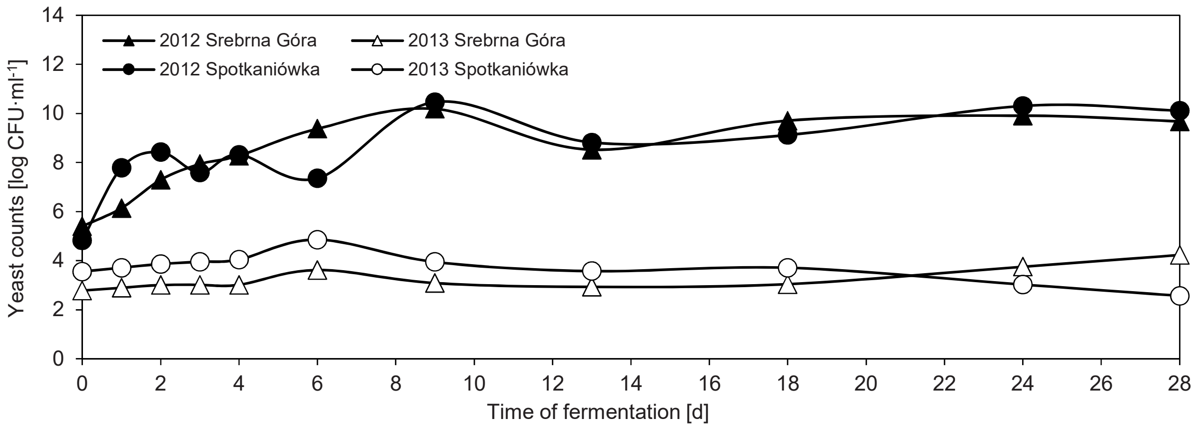

Fig. 1. Changes in the yeast counts in spontaneously fermented grape musts obtained from Hibernal variety, acquired from Srebrna Góra and Spotkaniówka vineyards.

Standard deviations for all data were lower than 5 %.

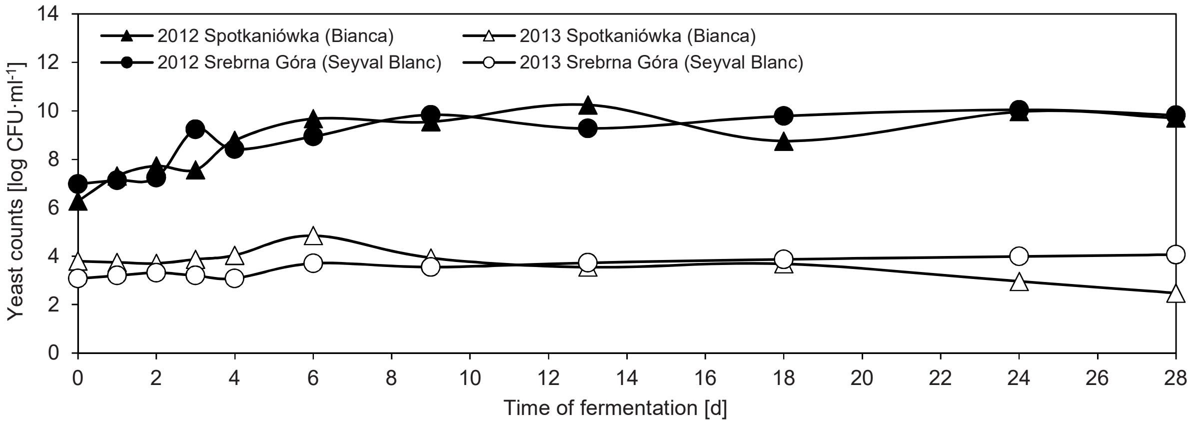

Fig. 2. Changes in the yeast counts in spontaneously fermented grape musts obtained from Bianca and Seyval Blanc varieties, acquired from Srebrna Góra and Spotkaniówka vineyards.

Standard deviations for all data were less than 5 %.

In 2012, an increase in the content of yeasts in juices obtained from fruits from both vineyards was recorded already after 24 h of fermentation (Fig. 1, Fig. 2). In the initial stage of the process, mainly aerobic strains are known to multiply, which are not very resistant to an elevated alcohol concentration [10]. In musts obtained from grapes from the Srebrna Góra vineyard, the growth of yeasts took place at the logarithmic rate until the 6th day of spontaneous fermentation. Then, yeast content remained constant. In musts obtained from grapes from the Spotkaniówka vineyard, an increase in the content of yeasts was observed until the 2nd day and then after the 6th day of the process. The slight decrease in the population of yeasts on the 3rd and 6th day could be the result of the slow death of more sensitive strains (Fig. 1, Fig. 2).

In all tested musts, yeasts reached a maximum level between the 4th and the 9th day of spontaneous fermentation. The rapid growth of microorganisms at this stage of the process is well described in the literature [11]. Then, there was a slight decrease in the content of yeasts in musts made from the Hibernal grape variety (Fig. 1, Fig. 2). With the progress of fermentation, the content of non-*Saccharomyces* yeasts decreases, as they give place to the *Saccharomyces* strains resistant to higher concentrations of alcohol [12]. However, *Kloeckera*, *Metschnikowia* or *Candida* may persist throughout the duration of the process, although their participation in the final stage is much lower [13], what was observed in this study. The 2013 season was characterized by a much smaller content of microorganisms in the analysed musts.

A small decrease in the yeast population on the 18th day of the fermentation of musts obtained from Bianca grapes (2012) and an increase in their numbers on subsequent days was observed. It could be the result of re-multiplication of non-*Saccharomyces* strains resistant to unfavourable conditions, when competition of *Saccharomyces* strains decreased (Fig. 2). Our results are in agreement with a previously published observation that some *Hanseniaspora* strains show aerobic growth, multiplying before or at the beginning of fermentation, while others are identified even in the final stage [14].

Hanseniaspora spp. belong to the most important non-*Saccharomyces* yeasts present during spontaneous wine fermentation, especially during its early stages [1]. Their content in juices obtained from Seyval Blanc grapes from the Srebrna Góra vineyard (2012) was at the concentration of 2.2×10^5 CFU·ml^{-1}. Slightly lower content was recorded in fresh pressed musts of Hibernal and Bianca variety fruits. In juices obtained from Seyval Blanc and Bianca variety grapes in 2013, no strains of the genus *Hanseniaspora* were found in the initial fermentation stage (Fig. 3, Fig. 4).

On the 2nd day of fermentation, the content of yeasts increased significantly in juices obtained from grapes from the 2012 season. The maximum yeast population was recorded between the 6th and the 13th day of spontaneous fermentation in samples from the 2012 season, and from the 4th to the 6th day of the process in 2013 (Fig. 3, Fig. 4).

Yeasts belonging to the genus *Kloeckera*/*Hanseniaspora*, including *K. apiculata*, *K. apis* and *K. javanica*, are characterized by lower fermentation activity and by the production of only low alcohol concentrations. However, some literature reports on benefits of the use of these microorganisms in mixed cultures in the fermentation process, as they could exhibit several beneficial properties. For example, *K. apiculata* strains, compared to *S. cerevisiae*, produces larger amounts of glycosidases and proteases, which are responsible for the production of compounds that determine the aroma and taste of the beverage [15].

In all the analysed samples, the content of *Hanseniaspora* yeasts decreased just after reaching its maximum level. At the end of spontaneous fermentation, they were not found in wines. These strains can account for 50–75 % of the total grape microbiota and, during the fermentation process, they can reach up to 99 % of the total yeast content [2]. The small amount of *Hanseniaspora* strains in analysed musts was connected with the lower total yeast content in the fruit in 2013, compared to the 2012 season. At the end of spontaneous fermentation, their presence was not confirmed. *Hanseniaspora* yeast has the ability to produce high levels of ethyl and amyl acetates, which directly affect the aroma of beverages. In addition to esters, the yeasts also synthesize glycerol and acetoin. The extent of production of these components is an individual feature of each yeast strain. When their content during late stages of fermentation is more than 10% of the total microbiota, they can be a risk factor adversely affecting the sensory characteristics of wine [16].

Physico-chemical characteristics of grape musts

The musts obtained from the tested varieties differed in their physico-chemical properties (Tab. 2). The concentration of total sugars ranged

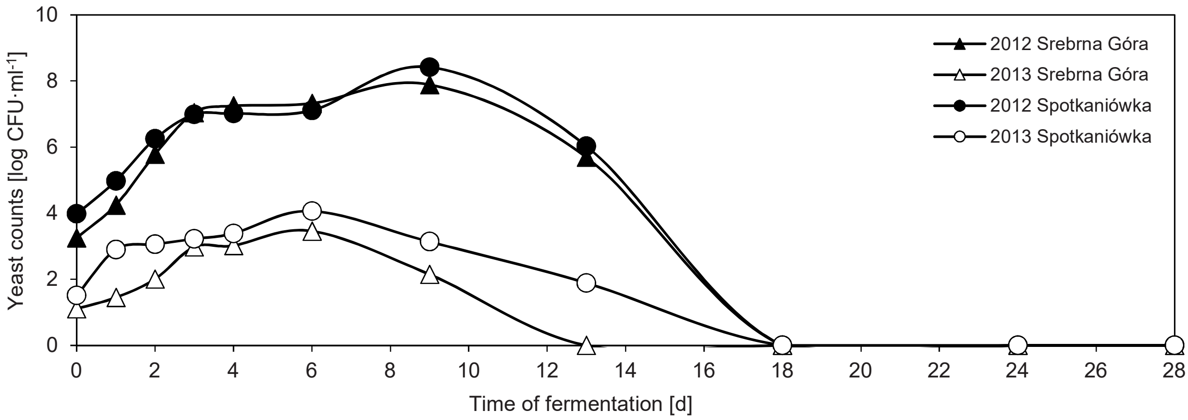

Fig. 3. Changes in counts of *Hanseniaspora* yeasts in spontaneously fermented grape musts obtained from Hibernal variety, acquired from Srebrna Góra and Spotkaniówka vineyards.

Standard deviations for all data were less than 5 %.

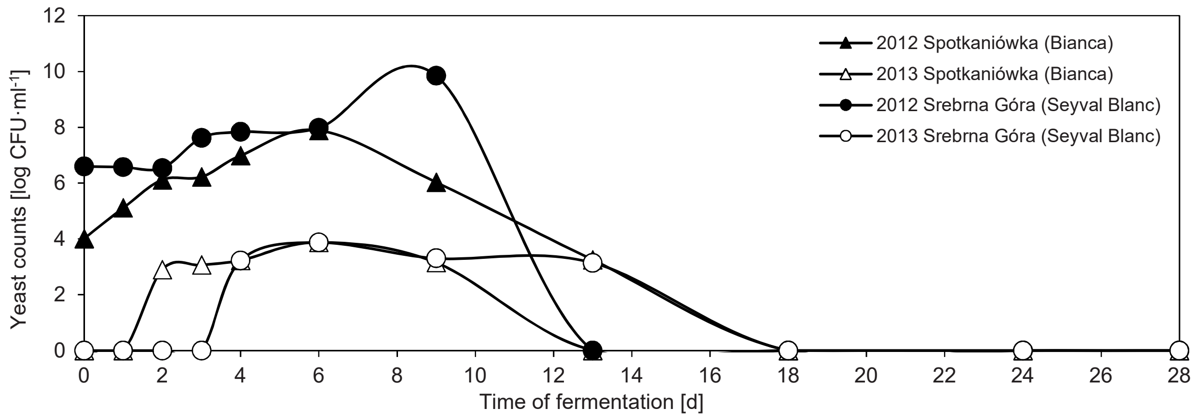

Fig. 4. Changes in the counts of *Hanseniaspora* yeasts in spontaneously fermented grape musts obtained from Bianca and Seyval Blanc varieties, acquired from Srebrna Góra and Spotkaniówka vineyards.

Standard deviations for all data were less than 5 %.

Tab. 2. Characterization of grape musts obtained from the Hibernal, Seyval Blanc and Bianca grape varieties.

Grape variety	Vineyard	Season	pH	Total acidity [g·l-1]	Extract [°Bx]	Sugars [g·l-1]
Hibernal	Srebrna Góra	2012	3.20 ± 0.00 e	8.33 ± 0.02 a	23.92 ± 0.12 d	239.20 ± 1.20 d
	Srebrna Góra	2013	2.91 ± 0.01 ab	12.40 ± 0.20 d	21.98 ± 0.20 c	219.80 ± 2.00 c
	Spotkaniówka	2012	3.01 ± 0.01 c	10.94 ± 0.25 c	21.10 ± 0.40 bc	211.00 ± 4.00 bc
	Spotkaniówka	2013	2.88 ± 0.01 a	14.88 ± 0.20 e	17.18 ± 0.32 e	171.80 ± 3.20 e
Seyval B.	Srebrna Góra	2012	3.31 ± 0.01 f	8.39 ± 0.01 a	24.54 ± 0.22 d	245.41 ± 2.21 d
	Srebrna Góra	2013	2.98 ± 0.02 c	11.45 ± 0.05 c	20.10 ± 0.30 a	201.00 ± 3.00 a
Bianca	Spotkaniówka	2012	2.93 ± 0.10 b	9.87 ± 0.28 b	20.20 ± 0.20 ab	202.00 ± 2.00 ab
	Spotkaniówka	2013	3.07 ± 0.15 d	10.17 ± 0.02 b	18.38 ± 0.23 f	183.80 ± 2.30 f

Values are expressed as mean ± standard deviation. Total acidity is expressed as grams of malic acid. The results marked with the same letters do not differ significantly ($p > 0.05$).

from 171.8 g·l^{-1} (in Hibernal musts from the Spotkaniówka vineyard, 2013) to 245.4 g·l^{-1} (in Seyval Blanc musts from the Srebrna Góra vineyard, 2012). In 2013, the concentrations of total sugars in grapes were lower than in the 2012 season. The exception was the Hibernal must (Srebrna Góra, 2013). The Hibernal must (2013) was also characterized by a relatively high total acidity (12.40 g·l^{-1}, 14.88 g·l^{-1}). The pH values of wines were from 2.88 to 3.31 (Tab. 2).

Yeast identification

A total of 152 (in 2012) and 78 (in 2013) yeast isolates were isolated randomly at different stages of the spontaneous fermentation from all tested varieties. RAPD-PCR restriction fragments length polymorphism (PCR-RFLP) analysis were performed to differentiate yeasts and reduce the number of samples taken for further analysis. All isolates were classified into 11 groups characterized by distinct RAPD electrophoretic patterns (Tab. 3) and, after 5.8S-ITS rRNA gene region sequencing, identified as members of 6 different species. Most frequently, the identified strains belonged to species *Metschnikowia pulcherrima*, *Hanseniaspora uvarum* and *Saccharomyces cerevisiae*. *Wickerhamomyces anomalus*, *Kluyveromyces lactis* and *Zygoascus meyerae* strains were also distinguished (Tab. 3). It is worth mentioning that musts and white wines are characterized by lower diversity of yeasts compared to red ones, which is associated with their lower pH, creating less favourable conditions for the growth of these microorganisms [17]. Analogous studies were conducted on grape must obtained from red grape varietes of Rondo and Regent [9]. A significantly higher species diversity was determined in the spontaneously fermented red grape musts. In addition to the above mentioned microorganisms, *Candida railenensis*, *C. oleophila*, *Nakazawaea ishiwadae* and *Pichia membranifaciens* were identified.

Tab. 4–7 show the percentages of yeast strains isolated throughout spontaneous fermentation of musts of Hibernal, Bianca and Seyval Blanc grape varieties in two consecutive years. *M. pulcherrima*, *H. uvarum* and *S. cerevisiae* strains dominated, being identified at each stage of spontaneous fermentation. Research carried out by other scientists showed that the strains *H. uvarum* and *M. pulcherrima* were among the most common yeasts present on grapes [18]. *W. anomalus* species were isolated only from the Spotkaniówka and Srebrna Góra vineyard from the fermented Hibernal must, their presence being not recorded in Bianca and Seyval Blanc musts. The presence of *Z. meyerae* was found only in musts from the Spotkaniówka vineyard. *Kluyveromyces lactis* strains were present also in musts from the Spotkaniówka vineyard, but only from the Hibernal variety (Tab. 3–7).

Among all identified strains, *M. pulcherrima* strains were dominant. These microorganisms occurred frequently during the whole fermentation process in samples from all vineyards (Tab. 3–7). Similar results were obtained by Bisson and Joseph [19]. Some research indicated a decrease in the number of these strains in grape musts after 100–130 h of spontaneous fermentation. After 10 days of the process, they are no longer detected [20]. Díaz et al. [21] reported the occurrence of the yeast *M. pulcherrima* in fermenting grape juice for at least 5 days longer. Along with the cultures of *Hanseniaspora*, *Candida* and *Pichia*, they constitute the microbiota present on fruits both in Asian countries and in Europe [22].

The 2013 season was characterized by cool temperatures as well as higher than average precipitation in summer. These atmospheric conditions favour the development of *M. pulcherrima*

Tab. 3. Yeast species identified on the basis of RFLP and ITS sequencing.

Season	Isolate	5.8S-ITS length [bp]	Restriction fragments [bp]			Species identification based on sequencing		Accession No.
			Hinf I	Hae III	Cfo I	Species	Sequence identity [%]	
2012	135	700	350+200+150	700	500+320	*Zygoascus meyerae*	98	MG970696
	80	390	200	300+200	250+100	*Metschnikowia pulcherrima*	98	MG971250
	93	390	220	350+300+200	220+120	*Metschnikowia pulcherrima* [a]	98	MG971249
	123	750	300+280	350	250	*Hanseniaspora uvarum* [b]	99	MG971252
	107	650	315	310	570+60	*Wickerhamomyces anomalus*	99	MG971261
	228	880	400	350+250+200+150	350	*Saccharomyces cerevisiae* [c]	98	MH020215
2013	81	390	200	300+180	200	*Metschnikowia pulcherrima*	99	MG971260
	109	390	390+220	300+200	300+180	*Metschnikowia pulcherrima*	99	MG971253
	23	750	320	380+220	750	*Hanseniaspora uvarum*	98	MG971266
	37	390	220	300	250	*Metschnikowia pulcherrima* [d]	99	MG971257
	92	740	250+200+100	650	300+180	*Kluyveromyces lactis* [e]	99	MG971263

Species were isolated from spontaneously fermented musts: a – Bianca and Hibernal grape varieties (Spotkaniówka vineyard) and Seyval Blanc variety (Srebrna Góra vineyard), b – Hibernal variety (Spotkaniówka and Srebrna Góra vineyard), c – Bianca grape variety (Spotkaniówka vineyard), Hibernal variety (Spotkaniówka and Srebrna Góra vineyard) and Seyval Blanc variety (Srebrna Góra vineyard), d – Seyval Blanc grape variety (Srebrna Góra vineyard) and Hibernal and Bianca varieties (Spotkaniówka vineyard), e – Hibernal variety (Spotkaniówka vineyard).

Tab. 4. Distribution of yeast strains isolated from various stages of spontaneous fermentation of the Hibernal (Spotkaniówka vineyard) grape musts.

Species	Accession No.	Content of yeast strains [%]																					
		Sampling days in season 2012											Sampling days in season 2013										
		0	1	2	3	4	6	9	13	18	24	28	0	1	2	3	4	6	9	13	18	24	28
Hanseniaspora uvarum	MG971252	20	15	20	30	25	20	15	10	5			20		20	10	20	20	40	30	30		
Hanseniaspora uvarum	MG971266		10	20	20									20	10	10		10					
Kluyveromyces lactis	MG971263														20	10	10	10					
Metschnikowia pulcherrima	MG971249	10	15	10	10	5	20	25	40	10	20	10		10		10		10					
Metschnikowia pulcherrima	MG971250	30	30	10	20	10	10	20	30	15	10	20	20	30					10	10	30	40	40
Metschnikowia pulcherrima	MG971253	30	10		10	10	20	20		50	10		20	10	10	20	10	10		20		10	
Metschnikowia pulcherrima	MG971257																						
Metschnikowia pulcherrima	MG971260	10		10		10					20	20	20	10	10	20	20	10	50	40	20	10	10
Saccharomyces cerevisiae	MH020215								20	20	40	50									20	40	50
Wickerhamomyces anomalus	MG971261		20	20		20	20	20					20		10		20	10					
Zygoascus meyerae	MG970696			10	10	20	10							10	20	20	20	20					

Tab. 5. Distribution of yeast strains isolated from various stages of spontaneous fermentation of the Hibernal (Srebrna Góra vineyard) grape musts.

Species	Accession No.	Content of yeast strains [%]																					
		Sampling days in season 2012											Sampling days in season 2013										
		0	1	2	3	4	6	9	13	18	24	28	0	1	2	3	4	6	9	13	18	24	28
Hanseniaspora uvarum	MG971252	10	20	30	30	20	40	20					20	10	20	20	30	40	20	10			
Hanseniaspora uvarum	MG971266	10	20	20	10	10							20										
Kluyveromyces lactis	MG971263																						
Metschnikowia pulcherrima	MG971249																						
Metschnikowia pulcherrima	MG971250	20	20	10	30	20	30	60	50	50	20	30	20		20	40	10	20	50	20	60	10	20
Metschnikowia pulcherrima	MG971253	40	30	10	10	40	10	20	40	20	20	10	20	50	30		20	20		20	20	30	20
Metschnikowia pulcherrima	MG971257																						
Metschnikowia pulcherrima	MG971260	20		10	10		20				10			30	10	40	30	20	30	40		20	10
Saccharomyces cerevisiae	MH020215								10	30	40	50								10	20	40	50
Wickerhamomyces anomalus	MG971261		10	20	10	10					10	10	20	10	20		10						
Zygoascus meyerae	MG970696																						

Tab. 6. Distribution of yeast strains isolated from various stages of spontaneous fermentation of the Bianca (Spotkaniówka vineyard) grape musts.

Species	Accession No.	Content of yeast strains [%]																					
		Sampling days in season 2012											Sampling days in season 2013										
		0	1	2	3	4	6	9	13	18	24	28	0	1	2	3	4	6	9	13	18	24	28
Hanseniaspora uvarum	MG971252																						
Hanseniaspora uvarum	MG971266	10	30		20	20	10	30	10				20	20	10	10	10	10					
Kluyveromyces lactis	MG971263																						
Metschnikowia pulcherrima	MG971249	10		10	20		10						10		20								
Metschnikowia pulcherrima	MG971250	20	30	10	20	30	30	20	30	40	30	20	20	20	20	50	30	40	40	50	20		10
Metschnikowia pulcherrima	MG971253	15	10	20	10		30	30	20	20			30	20	20	20	20	10		30	40	60	30
Metschnikowia pulcherrima	MG971257	20	15	10	20			10	10					10	10			10					
Metschnikowia pulcherrima	MG971260	25	15	30		30	20	10	20	20	30	30	20	30	20		20		50	20	20		
Saccharomyces cerevisiae	MH020215								10	20	40	50									20	40	60
Wickerhamomyces anomalus	MG971261																						
Zygoascus meyerae	MG970696			10	10	20										20	20	30	10				

Tab. 7. Distribution of yeast strains isolated from various stages of spontaneous fermentation of the Seyval Blanc (Srebrna Góra vineyard) grape musts.

Species	Accession No.	Content of yeast strains [%]																					
		Sampling days in season 2012											Sampling days in season 2013										
		0	1	2	3	4	6	9	13	18	24	28	0	1	2	3	4	6	9	13	18	24	28
Hanseniaspora uvarum	MG971252																						
Hanseniaspora uvarum	MG971266	20	20	20	20	30	40	20	10				30	30	30	40	20	40	40	20			
Kluyveromyces lactis	MG971263																						
Metschnikowia pulcherrima	MG971249		10	10		10							10	20	10		20						
Metschnikowia pulcherrima	MG971250	30	10	30		20	10	30	30	20	20	10	30	30	10	50	40		10		30	20	10
Metschnikowia pulcherrima	MG971253	20	20	20	20	10	20	20	30	20	30	30	30	20	30	10	20	20	30	30	40	40	20
Metschnikowia pulcherrima	MG971257	10	20	10	20	30	10		10	20	10												
Metschnikowia pulcherrima	MG971260	20	20	10	40		20	30	10	20	10				20			40	20	40	10	20	30
Saccharomyces cerevisiae	MH020215								10	20	30	60								10	20	20	40
Wickerhamomyces anomalus	MG971261																						
Zygoascus meyerae	MG970696																						

strains [13]. Yeasts are sensitive to environmental conditions and have relatively high nutritional requirements. Hence, the differences in the microbiota profile between vineyards and subsequent periods testify to the strong influence of climatic conditions on the abundance of microorganisms and their presence during spontaneous fermentation. This was also confirmed by our further research (data not yet published).

The species *W. anomalus*, formerly known as *Hansenula anomala*, *Candida pelliculosa* and *Pichia anomala* [23], naturally occurs in the grape must [21]. These yeasts are active in the early phase of fermentation. They can also cause spoilage of wine when they produce too high levels of acetic acid and ethyl acetate [24]. This species shows strong growth in the grape must. However, it is inhibited by *S. cerevisiae* strains [25]. *W. anomalus* gives a unique aromatic profile to wines by producing acetate esters such as ethyl acetate, ethyl caproate and ethyl caprylate [26]. These compounds are very beneficial for the aroma of wine. Wines obtained with participation of *W. anomalus* are more preferred by the tasters, compared to those obtained with a *S. cerevisiae* mono-culture [27]. Similar results were obtained for ciders [28]. Moreover, it was found that *W. anomalus* strains secreted the toxin Pikt against *Dekkera* ssp. (anamorph *Brettanomyces*) [29]. This species is sensitive to SO_2 [27], in contrast to other wine yeasts that are able to survive in this environment. It exhibits tolerance to sugar and oxygen [23]. It is advantageous, because yeast cells synthesize reactive oxygen species (ROS) when the amount of available oxygen is limited [30]. This species shows high physiological variability [23]. The *W. anomalus* strain was isolated from various samples in the initial stage of fermentation (Tab. 3–7), as well as at the end of fermentation of the Hibernal musts obtained from the Srebrna Góra vineyard (Tab. 7). Previously, it was detected in a South African red grape must [6].

Non-*Saccharomyces* yeasts of *Wickerhamomyces*, *Kloeckera*, *Candida*, *Debaryomyces*, *Rhodotorula*, *Metschnikowia*, *Hanseniaspora* and *Kluyveromyces* species can produce hydrolytic exoenzymes (esterase, lipase, glycosidase, glucanase, pectinase, amylase and protease) that interact with grape components [31]. For example, glycosidic hydrolases can release aromatic compounds into the grape must from their odourless glycoside precursors [32]. Other strains produce pectinolytic enzymes that could clarify the grape must and thus replace fungal enzymes that are currently used in the wine industry [31]. Yeasts secreting proteolytic enzymes are also of great biotechnological importance in wine protein haze prevention. They can be added as starter cultures to the grape must [31]. Studies showed a high protease activity of *Wickerhamomyces* yeasts isolated from oenological systems [33]. Extracellular protease activity was also observed in *M. pulcherrima* and *Z. meyerae* yeasts. Genes coding for extracellular proteases in these microorganisms have been isolated and they were found to be active against grape components that cause wine turbidity. Furthermore, their activity against grape proteins can release assimilable nitrogen in the form of amino acids and change the aromatic profile of the wine [34]. *Z. meyerae* strains were isolated in the initial stage of spontaneous fermentation of must obtained from Bianca grapes from the Spotkaniówka vineyard (Tab. 7). They were also detected in the Hibernal musts from the same vineyard. However, they were not identified in spontaneously fermented musts obtained from grapes from the Srebrna Góra vineyard (Tab. 3–7).

Strains from the genus *Saccharomyces* were the second largest group of yeasts isolated during spontaneous fermentation of grape musts. These yeasts are the best known in terms of their structure, physiology and metabolism. They are rarely identified on grape vine fruits but frequently on the contact surfaces of the vineyard [35]. During spontaneous fermentation, they suppress other cultures and take over the environment [36]. All isolates from this genus were classified as *S. cerevisiae* species. In the conducted studies, the occurrence of *S.cerevisiae* strains was recorded from the 9th day of the process (Tab. 4–7). Rapid multiplication of these microorganisms with devitalization of the *Kloeckera/Hanseniaspora* population was observed. In studies carried out by Combina et al. [37], strains from the genus *Saccharomyces* were recorded as early as on the 2nd day of spontaneous fermentation of grape juice, and on the 30th day their proportion reached 100 %. It is well established that, as the fermentation process proceeds, the proportion of non-*Saccharomyces* yeasts decreases in favour of *Saccharomyces* species. It is related to the tolerance of the latter to increased alcohol concentrations and to the secretion of killer-like compounds by them [38].

CONCLUSIONS

The quantity of yeasts colonizing grapevine fruits is a function of many external factors. The most important include the physical and chemical properties of the surrounding environment, climatic conditions and the applied agrotechnical treatments. The grapes used in this study came from two different vineyards and the influence of the above factors on the quantitative and qualitative diversity of microorganisms was found to be significant. It was found that the grape variety plays an important role in shaping the yeast microbiota. In the 2012 season, there was a higher yeast content in fresh musts compared to 2013. The musts obtained from the grapes from the Spotkaniówka vineyard were characterized by a higher yeast content compared to those obtained from the Srebrna Góra vineyard. It could be related to the microclimatic conditions in the vineyard. A slight increase in the content of yeasts at the end of spontaneous fermentation of all musts obtained in 2012 was observed. Probably it resulted from the development of species resistant to increased ethanol concentrations and to the use of other nutrient resources present in the must. Interestingly, *Z. meyerae* and *K. lactis* strains were isolated exclusively from fermented grape musts obtained from grapes from the Spotkaniówka vineyard. Also, the presence of *Z. meyerae* was found in musts obtained from the 'Rondo' variety from this vineyard in 2012 [9]. Their presence may be related to the microclimatic conditions prevailing in the vineyard.

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