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YEAST DIVERSITY IN NEW, STILL FERMENTING WINE "FEDERWEISSER"

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ABSTRACT

The aim of this study was to isolate and identify yeasts in different new wine “federweisser” samples. We collected the samples at the end of the August 2015 and in the middle of the September 2015. Used 15 new wine samples in this study (5 white and 10 red) were from the local Slovak winemakers. Irsai Oliver (3), Moravian Muscat (2), Agria/Turan (1), Dornfelder (3), Blue Frankish (3), Pinot Noir (1) and Saint Laurent (2). Three cultivation media were used for detection of yeasts in “federweisser” samples. Malt extract agar base (MEA), Wort agar (WA) and Wild yeast medium (WYM) were used for the cultivation of yeasts. Cultivation was performed by spread plate method. Ethanol/formic acid extraction procedure was used for preparation of samples. MALDI-TOF Mass Spectrometer (Microflex LT/SH) (Bruker Daltonics, Germany) was used for the identification of yeasts. We identified seven different strains of *Saccharomyces cerevisiae* (23; 70%), two strains of *Kloeckera apiculata* [teleomorph *Hanseniaspora uvarum*] (7; 21%), and one strain of *Pichia kluyveri* (1; 3%), *Pichia occidentalis* [anamorph *Candida sorbosa*] (1; 3%) and *Metschnikowia pulcherrima* (1; 3%) in 15 new wine “federweisser” samples. *Saccharomyces cerevisiae* was dominant species in each new wine sample, and formed creamy convex colonies with circular edge. *Metschnikowia pulcherrima* formed convex to pulvinate, circular white-pink colored colonies, *Kloeckera apiculata* formed flat, circular smooth colonies with turquoise center with gray edge, *Pichia occidentalis* formed irregular pulvinate light-cream colored colonies, and *Pichia kluyveri* formed turquoise, convex, undulate and smooth colonies on Malt extract agar base with bromocresol green.

Keywords: new wine; yeasts; *Saccharomyces cerevisiae*; MALDI-TOF MS

INTRODUCTION

Federweisser wine is grape must which is just undergoing the process of fermentation. Grape must is the juice of the wine grapes which is gained after the pressing of grapes. After corresponding treatment and storing, the must would become wine after finishing the process of fermentation. Because of this, Federweisser is not specially produced as some kind of drink but as an early product of wine production. The fermentation causes the splitting of the fructose of the grapes in alcohol and carbon dioxide. Because of the yeasts and bacteria in the must, the fermentation goes on very quickly. That is why Federweisser is drinkable only a couple of days. But the cool storage can lengthen the process of fermentation. In the refrigerator, Federweisser can be kept about 10 days. The grape must is considered "Federweisser" wine as soon as the alcohol concentration is about 4 to 6%. At the beginning, it tastes quite sweet. During the process of fermentation, the sweetness subsides. Due to the concentration of carbon dioxide, Federweisser tastes very prickly and tangy. Because of the high carbon dioxide concentration, a corking or air tight closure of the Federweisser is not possible. Especially in the past, this caused a transportation problem. Federweisser could only be offered regionally and was limited. Grape must is inoculated with a pure culture of yeasts (*S. cerevisiae*), usually 10-20 g.100 L^{-1} of must. Federweisser is very good for cold or warm drinking **(Malík et al., 2012)**.

Yeasts are found throughout nature. However, they do not occur randomly, but are found in specific habitats where different species form communities **(Lachance and Starmer, 1998)**. Within the winemaking environment (habitat), the vineyard (grape surfaces) and cellar (equipment surfaces and must) can be considered specialized niches where the wine related yeasts can form communities **(Polsinelli et al., 1996)**. The yeast species found in different niches associated with grape growth (vineyards) and wine production (wineries, grape must, fermentation and wine) can be arbitrarily divided into two groups, i.e. the *Saccharomyces* group and the non-*Saccharomyces* group. The *Saccharomyces* group with its primary representative, *Saccharomyces cerevisiae*, is present on grape skins in low numbers **(Rankine, 1972; Török et al., 1996, König et al., 2009)**, and on winery equipment and in fermenting must in greater numbers **(Fugelsang et al., 2007)**. Non-*Saccharomyces* yeast are part of the natural microbiota present on grapes, and harvesting and winemaking equipment, and are present at least during the early stages of fermentation **(Fleet and Heard, 1993; Renouf et al., 2005, 2007)**. While generally incapable of completing alcoholic fermentation, their application in co-inoculation or sequential inoculation with *S. cerevisiae* is increasingly popular **(Ciani et al., 2006; Ciani and Maccarelli, 1998; Comitini et al., 2011; Jolly et al., 2006; Soden et al., 2000)**, particularly for their effects on wine composition, flavour and aroma **(Benito et al., 2011; Ciani et al., 2006; Comitini et al., 2011; Cordero Otero et al., 2003; Di Maio et al., 2012;**

Domizio et al., 2011; Garcia et al., 2002; Jolly et al., 2006, 2014; Magyar and Toth, 2011; Morata et al., 2012; Soden et al., 2000; Toro and Vazquez, 2002).

The fermentation of grape must is a complex microbiological process that involves interactions between yeasts, bacteria, and filamentous fungi **(Fleet, 2007; Fugelsang and Edwards, 2007)**. Yeasts, which play a central role in the winemaking process, are unicellular fungi that reproduce by budding **(Ribéreau-Gayon et al., 2006)**. More than 100 yeast species have been isolated from grapes, must and wine **(König et al., 2009)**. The predominant species on the grape is *Kloeckera apiculata*, which may represent more than 50% of the flora obtained from the fruit **(Fugelsang and Edwards, 2007)**. Other species of obligate aerobic or weakly fermentative yeasts with very limited alcohol tolerance may also be found in lesser proportions. These belong to the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, and *Rhodotorula* **(Fleet and Heard, 1993; Ribéreau-Gayon et al., 2006)**. The growth of these species, known collectively as non-*Saccharomyces* yeasts (or wild yeasts), is limited to the first 2 or 3 days of fermentation, after which they die as a result of ethanol toxicity. As these yeasts disappear, highly fermentative strains of the species *Saccharomyces cerevisiae* and *Saccharomyces bayanus* begin to multiply until they become solely responsible for alcoholic fermentation. The yeasts present in the must during the first few hours after filling the tanks belong to the same genera as those found on the grapes, predominantly *Kloeckera* (*Hanseniaspora*). In these spontaneous vinification conditions, *Saccharomyces* yeasts begin to develop after around 20h and are present alongside the grape-derived yeast flora. After 3rd or 4th day of fermentation, *Saccharomyces* yeasts predominate and are ultimately responsible for alcoholic fermentation **(Ribéreau-Gayon et al., 2006)**. This change in the yeast population is linked to the increasing presence of ethanol, the anaerobic conditions, and the use of sulfites during harvesting and in the must, the concentration of sugar, and the greater tolerance of high temperatures shown by *S. cerevisiae* compared with other yeasts **(Fleet and Heard, 1993; Fleet, 2007)**. *S. cerevisiae* comprises numerous strains with varying biotechnological properties **(Ribéreau-Gayon et al., 2006)**.

The aim of this study was to isolate and identify yeasts in different Slovak new wine "federweisser" samples.

MATERIAL AND METHODOLOGY

Federweisser samples, Spread plate method and Cultivation media

Samples of new wine "federweisser" were collected at the end of the August 2015 and in the middle of the September 2015 from local Slovak winemakers. Samples (apx.100 mL) were collected into 200 mL sterile plastic bottles with screw caps, and immediately stored at 8 ±1 °C in refrigerator. Bottle caps have been released, because the carbon dioxide (CO_2) was still produced by yeasts. Collected and stored samples (No. 15) were diluted with sterile physiological saline (0.85%), and dilution 10^{-4} and 10^{-5} were used for next analysis. 100 µL each dilution (10^{-4}, 10^{-5}) was placed on the surface of solidified agar media. The spread plate method was used for isolation of yeasts in federweisser samples. Samples were obtained from white (5) and red new wines (10). Irsai Oliver (3), Moravian Muscat (2), Agria/Turan (1), Dornfelder (3), Blue Frankish (3), Pinot Noir (1) and Saint Laurent (2). Three cultivation media were used for detection of yeasts in federweisser samples. Malt extract agar base (MEA) (BioMark™, India); Wort agar (HiMedia®, India) and Wild Yeast medium (HiMedia®, India). MEA has been enriched with glucose (CentralChem®, Slovakia) (50 g.L^{-1}), yeast extract (Conda, Spain) (3 g.L^{-1}) and acid base indicator bromocresol green (Sigma-Aldrich®, USA) (0.020 g.L^{-1}) (pH 3.8-5.4 yellow to blue). Yeasts were cultivated on Petri dishes at 25 °C for 5 days in aerobic conditions.

Identification of yeasts

We used MALDI-TOF Mass Spectrometer (Bruker Daltonics, Germany) for identification of yeasts isolated from federweisser samples. After incubation of yeasts at 25 °C for 5 days, isolated colonies were picked and suspended in 300 µL of sterile distilled water and mixed thoroughly. 900 µL of absolute ethanol was added. The mixture was centrifuged at 13 000 × g for 2 min. After the supernatant was discarded, the pellet was centrifuged again. Residual ethanol was completely removed by pipetting and the pellet was allowed to dry at room temperature. Subsequently 10 µL of formic acid (70%) was added and mixed with the pellet with a sterile toothpick. Next, 10 µL of acetonitrile (100%) was added and mixed thoroughly. The solution was centrifuged at maximum speed for 2 minutes again, and 1 µL of the supernatant was spotted on a polished MALDI target plate (Bruker Daltonics, Germany). Immediately after drying 1 µL of the matrix solution was added to each spot and allowed to air dry. The matrix used was a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) (Bruker Daltonics, Germany) dissolved in 50% acetonitrile with 0.025% trifluoroacetic acid (TFA). The matrix solution preparation (2.5 mg of HCCA) contains 500 µL of acetonitrile, 475 µL of ultra-pure water and 25 µL of trifluoroacetic acid. Next added 250 µL of this solution to the 2.5 mg of HCCA. Samples were then processed in the MALDI-TOF MS (Microflex LT/SH, Bruker Daltonics, Germany) with flex Control software and results obtained with Real-time Classification software (RTC) by used database "Taxonomy" (Bruker Daltonics, Germany).

RESULTS AND DISCUSSION

After cultivation time, we obtained results from number of CFU (colony forming unit) in 100 µL of new wine sample of each used decimal dilutions. For better interpretation of results logarithmic conversion was applied on numerical results. Natural logarithm (Log_e) was used in Microsoft® Office Excel program by function LN. The highest number of yeasts cultivated on malt extract agar (MEA) was found in sample number thirteen Pinot Noir 6.43 log CFU.100 µL^{-1} and the lowest number of yeasts cultivated on MEA was present in the third sample Moravian Muscat 4.62 log CFU.100 µL^{-1}. The highest number of yeasts cultivated on Wort agar (WA) was found also in sample number 13 Pinot Noir 6.39 log CFU.100 µL^{-1}, but the lowest number of yeasts

Table 1 Number of yeasts in federweisser samples in log CFU.100 μL^{-1}.

	Cultivation media	MEA		WA		WYM	
No.	**Variety**	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-4}	10^{-5}
1.	*Agria/Turan*	6.12	5.29	6.34	5.74	6.04	5.39
2.	*Irsai Oliver*	6.07	5.35	6.27	5.69	6.00	4.75
3.	*Moravian Muscat*	5.91	4.62	6.07	5.57	6.03	5.08
4.	*Irsai Oliver*	6.32	5.89	6.18	5.38	5.74	5.51
5.	*Blue Frankish*	6.30	ND	6.20	ND	5.63	ND
6.	*Irsai Oliver*	6.27	5.81	6.28	5.54	6.29	6.05
7.	*Blue Frankish*	6.31	ND	6.25	ND	6.33	ND
8.	*Moravian Muscat*	6.38	5.98	6.30	5.78	5.70	5.24
9.	*Blue Frankish*	6.37	6.06	6.03	5.75	5.82	5.61
10.	*Dornfelder*	6.39	6.09	6.28	5.76	5.66	5.12
11.	*Saint Laurent*	5.98	5.07	6.18	5.77	4.25	2.89
12.	*Dornfelder*	6.23	5.86	6.24	5.75	5.56	4.57
13.	*Pinot Noir*	6.43	6.14	6.39	6.18	ND	ND
14.	*Saint Laurent*	6.51	6.19	6.29	5.77	5.11	4.72
15.	*Dornfelder*	6.01	5.84	5.92	5.65	5.25	4.20

NOTE: **MEA**: Malt extract agar, **WA**: Wort agar, **WYM**: Wild yeast medium, **ND**: not detected.

cultivated on WA was present in the fourth sample Irsai Oliver 5.38 log CFU.100 μL^{-1}. The highest number of yeasts cultivated on Wild yeast medium (WYM) was found in sample number seven Blue Frankish 6.33 log CFU.100 μL^{-1} and the lowest number of yeasts cultivated on WYM was present in the fifteenth sample Dornfelder 4.20 log CFU.100 μL^{-1}. Table 1 contains results from microbiology of new wines obtained by spread plate method with used specific decimal dilutions 10^{-4} and 10^{-5}. Yeasts were countable at these two used dilutions. In this study we identified seven different strains of *Saccharomyces cerevisiae* (23), two strains of

Table 2 Yeast species in new wine "federweisser" samples.

No.	Variety	Species identified by MALDI-TOF MS
1.	**Turan/Agria**	*Saccharomyces cerevisiae* WS LLH
		Saccharomyces cerevisiae 991400574
		Kloeckera apiculata DSM 70788
2.	**Irsai Oliver**	*Saccharomyces cerevisiae* WS LLH
		Saccharomyces cerevisiae DSM 1334
3.	**Moravian Muscat**	*Saccharomyces cerevisiae* DSM 3798
		Saccharomyces cerevisiae WS LLH
		Kloeckera apiculata DSM 2768
4.	**Irsai Oliver**	*Saccharomyces cerevisiae* DSM 70868
5.	**Blue Frankish**	*Saccharomyces cerevisiae* DSM 1334
		Metschnikowia pulcherrima CBS 610NT
		Pichia kluyveri MY890_09
		Kloeckera apiculata DSM 2768
		Saccharomyces cerevisiae WS LLH
6.	**Irsai Oliver**	*Kloeckera apiculata* DSM 2768
		Pichia occidentalis CBS 1910
		Saccharomyces cerevisiae WS LLH
		Saccharomyces cerevisiae DSM 1334
7.	**Blue Frankish**	*Kloeckera apiculata* DSM 70788
		Saccharomyces cerevisiae DSM 1334
		Saccharomyces cerevisiae WS LLH
8.	**Moravian Muscat**	*Saccharomyces cerevisiae* WS LLH
		Kloeckera apiculata DSM 2768
		Saccharomyces cerevisiae CBS 1171
9.	**Blue Frankish**	*Kloeckera apiculata* DSM 2768
		Saccharomyces cerevisiae CBS 1171
10.	**Dornfelder**	*Saccharomyces cerevisiae* DSM 1334
11.	**Saint Laurent**	*Saccharomyces cerevisiae* DSM 1334
12.	**Dornfelder**	*Saccharomyces cerevisiae* DTY3
		Saccharomyces cerevisiae DSM 1334
13.	**Pinot Noir**	*Saccharomyces cerevisiae* DSM 70868
14.	**Saint Laurent**	*Saccharomyces cerevisiae* CBS 1171
15.	**Dornfelder**	*Saccharomyces cerevisiae* DSM 70868

Kloeckera apiculata (7), and one strain of *Pichia kluyveri* (1), *Pichia occidentalis* (1) and *Metschnikowia pulcherrima* (1) in fifteen federweisser samples. *Pichia kluyveri* was identified in Blue Frankish sample number five and *Pichia occidentalis* (anamorph *Candida sorbosa*) in sample number six (Irsai Oliver). We also identified one strain of *Metschnikowia pulcherrima* in sample number five (Blue Frankish).

The most common species in new wine samples was *Saccharomyces cerevisiae* and we identified seven different strains namely: DSM 1334, DSM 3798, DSM 70868, DTY3, CBS 1171, WS LLH and strain 991400574. Second most common species in new wine samples was *Kloeckera apiculata* (*Hanseniaspora uvarum*). *K. apiculata* was found in 7 new wine samples, two different strains (DSM 2768 and DSM 70788). Seven different strains of *Saccharomyces cerevisiae* was found in 15 new wine samples, what can be seen in Table 2.

S. cerevisiae is the most important yeast for wine production and is responsible for the metabolism of grape sugar to alcohol and CO_2. For these reasons *S. cerevisiae* is often simply referred to as "the wine yeast" **(Fleet, 1993; Pretorius et al., 1999; Swiegers and Pretorius, 2005)**. From all of identified yeasts, *Saccharomyces cerevisiae* was the dominant species, and we identified this species in all 15 new wine samples (70%). Grapes contain different species of yeast belongs to non-*Saccharomyces* yeasts such as *Kloeckera* (dominant genera), *Metschnikowia, Candida, Pichia, Rhodorotula, Aureobasidium* etc. *Saccharomyces* yeasts are not present in grape surface, or present in very low levels (less than 50 $CFU.mL^{-1}$) **(Prakitchaiwattana et al., 2004; Combina et al., 2005; Raspor et al., 2006; König et al., 2009)**.

When alcoholic fermentation starts non-*Saccharomyces* yeast population decrease. After the start of alcoholic fermentation when the ethanol concentration reaches 5 to 6% these yeast will be die **(Fugelsang et al., 2007)**. As fermentation progresses, the levels of these yeasts decrease, while that of *Saccharomyces* increases **(Fleet and Heard 1993)**. By the end of fermentation, *Saccharomyces* is the majority of the yeasts found, and often the only yeast isolated. New, still fermenting wine contains 4 to 6% ethanol and mostly contains only *Saccharomyces cerevisiae*, which is always predominant in new wines. But yeasts as *Kloeckera, Metschnikowia, Candida, Pichia* etc. can be identified in new wine samples in low populations. Some winemakers use commercial pure cultures and the others prefer to encourage the growth of some non-*Saccharomyces* yeasts early in alcoholic fermentation but eventually inoculate with *Saccharomyces* **(Fugelsang et al., 2007)**.

We identified except *S. cerevisiae* also *Kloeckera apiculata* in 7 new wine samples (21%) in lower population. Very interesting was that we isolated and identified only 3 another species of yeasts: *Metschnikowia pulcherrima* (3%), *Pichia kluyveri* (3%) and *Pichia occidentalis* (3%). In study **Kántor et al., (2015)** bromocresol green was used as a supplement also in Malt extract agar base (MEA) from BioMark™ (India). But in that study, cultivation media was not supplemented with yeast extract and glucose, only bromocresol green was added. After sterilization by autoclaving, that medium had a dark blue color. However in this study we supplemented Malt extract agar base (BioMark™, India) with yeast extract and glucose, and after sterilization by autoclaving had medium olive-green color. Malt extract agar base (BioMark™, India) contains only malt extract (30 $g.L^{-1}$), mycological peptone (5 $g.L^{-1}$) and agar (15 $g.L^{-1}$). Supplementation was desired in this case with glucose, yeast extract and bromocresol green. Yeasts grow very well in this modified medium, and bromocresol green is very helpful in differentiating of yeasts. Figure 1 shows the different colony morphology of 4 yeast species grown on MEA in sample number 5 (Blue Frankish). As you can see, the number of *Saccharomyces cerevisiae* was the highest, then *Kloeckera apiculata* and after that *Pichia kluyveri* and

Figure 1 Yeast species isolated from new wine "Federweisser" (Sample no. 5, dilution 10^{-4}).

only one colony on this petri dish belonged to *Metschnikowia pulcherrima*. *M. pulcherrima* produced maroon colored pigment called pulcherrimin, which was visible from the bottom of the petri dish. *Metschnikowia pulcherrima* formed convex to pulvinate, circular white-pink colonies. *Kloeckera apiculata* formed flat, circular smooth colonies with turquoise center with gray edge. *Pichia kluyveri* formed turquoise, convex, undulate and smooth colonies and *Pichia occidentalis* formed irregular pulvinate light-cream colonies.

CONCLUSION

In this study we isolated and identified yeast species in 15 Slovak new wine "Federweisser" samples. We identified the yeast isolates by MALDI-TOF mass spectrometry biotyper (Bruker Daltonics, Germany). The most dominant species was *Saccharomyces cerevisiae* which was isolated from all 15 new wine samples, which was a very good result. By mass spectrometry we identified 7 different strains of *S. cerevisiae*. The second most common species was *Kloeckera apiculata* (*Hanseniaspora uvarum*) found in 7 new wine samples (2 strains). We also identified other non-*Saccharomyces* yeasts such as *Metschnikowia pulcherrima* (1 strain), *Pichia occidentalis* (1 strain) and *Pichia kluyveri* (1 strain).

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