Determination of Spoilage Yeasts in Different Red and White Wines

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Abstract
The aim of this study was to isolate and identify yeast species in wine samples from five different countries, and determine the genera of spoilage yeasts. One hundred and thirty four (no. 134) wine samples (86 white and 48 red wine); (30 bottled, 104 tank wine) from five countries Slovakia (no. 53), Czech Republic (no. 17), Hungary (no. 6), Spain (no. 32) and Italy (no. 26) were used in this study. Yeasts were cultivated on Malt extract agar supplemented with bromocresol green. After cultivation, yeasts were identified on species level by MALDI-TOF mass spectrometry. We identify 10 yeasts genera from total of 285 isolates; *Saccharomyces* (97 isolates, 1 species), *Pichia* (120 isolates, 5 species), *Kregervanrija* (6 isolates, 1 species), *Zygosaccharomyces* (32 isolates, 2 species), *Candida* (13 isolates, 2 species), *Rhodotorula* (4 isolates, 1 species), *Wickerhamomyces* (7 isolates, 1 species) *Meyerozyma* (4 isolates, 1 species) and genera with only 1 isolate and species: *Torulaspora*, and *Cyberlindnera.*

Keywords: yeasts; wine samples; MALDI-TOF MS.

1. Introduction

In fermented alcoholic beverages, the concept of spoilage yeast has a more complex meaning than in non-fermented foods, where any yeast able to change food sensorial characteristics can be regarded as a ‘‘spoilage yeast.’’ In fermented drinks or foods, yeast activity is essential during the fermenting process. In the wine industry where alcoholic fermentation occurs in the presence of many yeast species and bacteria (mainly lactic and acetic), it is very difficult to draw a line between beneficial fermenting activity and spoilage activity. For this reason, spoilage yeasts are rarely sought during wine fermentation, but during storage or aging and during the bottling process [1]. Important wine spoiling yeasts genera are *Candida*, *Pichia*, *Brettanomyces* and *Zygosaccharomyces*. The last two genera of yeast are as ethanol tolerant as *Saccharomyces cerevisiae* and may be found in bottled wine. Their presence is influenced by the degree of filtration that precedes bottling and cellar hygiene during bottling. *Zygosaccharomyces* species are considered as spoilage yeast due to high production of volatile acidity (high amount of acetic acid) [1-3]. Undoubtedly, yeasts belonging to the genus *Brettanomyces* (teleomorph *Dekkera*) represent a major problem in the wine industry due to their high capability to produce volatile phenols (ethyl and vinyl phenols) [1, 4, 5]. Some *Pichia* species are able to produce the same volatile phenols in wine like *Brettanomyces.* Species which are able to produce volatile phenols are *Pichia manshurica* and *Pichia membranifaciens* [6]. The common spoilage effects are film formation in stored wines, cloudiness or haziness, sediments, gas production in bottled wines and off odors and off-tastes at all stages of wine production [1].

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The aim of this study was to isolate and identify yeast species in wine samples from five different countries, and determine the genera of spoilage yeasts.

2. Material and methods

Wine samples: One hundred and thirty four (no. 134) wine samples (86 white and 48 red wine); (30 bottled, 104 tank wine) from five countries Slovakia (no. 53), Czech Republic (no. 17), Hungary (no. 6), Spain (no. 32) and Italy (no. 26) were used in this study. Approximately 200 mL of each wine sample was collected into sterile plastic bottle with screw cap. Wines were stored at 8±2°C to microbiological analysis.

Microbiological examination and cultivation conditions: Collected and stored samples (no. 134) were diluted with sterile physiological saline (0.85%), ~~and~~ dilution 10^{-2} and 10^{-3} were used. The spread plate method was used for isolation of yeasts in wine samples and 0.1 mL of each dilution (10^{-2}, 10^{-3}) was placed on the surface of the solidified cultivation medium. Yeast cultivated on Malt extract agar base (MEA) (BioMark®, India) supplemented with glucose (20 g/L) (Centralchem®, Slovakia), yeast extract (3 g/L) (Conda, Spain) and bromocresol green (0.020 g/L) (Centralchem®, Slovakia). Yeasts were cultivated on Petri dishes at 25 °C for 5 days in aerobic conditions and randomly picked colonies (macroscopic morphological differences) were re-streaked into new agar plate. Then the proteins were extracted from fresh yeast colonies.

MALDI-TOF Mass Spectrometry: MALDI-TOF MS model Microflex LT/SH biotyper (Bruker Daltonics, Germany, Bremen) was used for identification of yeasts isolated from different red and white wine samples. After incubation (at 25 °C for 5 days), isolated colonies were picked up form cultivation media (MEA) and suspended in 300 µL of sterile distilled water and mixed thoroughly. 900 µL of absolute ethanol (99 %, Sigma-Aldrich, USA) was added. The mixture was centrifuged at 13 000 × g for 2 min. After that, supernatant was discarded, and pellet was centrifuged again. Residual ethanol was completely removed by pipetting and the pellet was allowed to dry at a room temperature. Subsequently 10 µL of formic acid (70 %, Sigma-Aldrich, USA) was added and mixed with the pellet with a sterile toothpick. Next, 10 µL of acetonitrile (100 %, Sigma-Aldrich, USA) 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 HCCA: α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Germany) dissolved in 50 % acetonitrile with 0.025 % trifluoroacetic acid (TFA) (100 %, Sigma-Aldrich, USA). The matrix solution preparation (2.5 mg of HCCA) contains 500 µL of acetonitrile, 475 µL of ultrapure water and 25 µL of trifluoroacetic acid. Next it was 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, Bremen) with flex Control software v3.4 and results obtained with Realtime Classification software (RTC) v3.1 (Bruker Daltonics, Germany). Each spectrum was obtained by averaging 240 laser shots acquired in the automatic mode at the minimum laser power necessary for ionization of the samples. The spectra have been analyzed in an m/z range of 2 to 20 kDa [7-9].

3. Results and discussion

We identified 10 genera of yeast and 16 yeast species using MALDI-TOF MS biotyper. The most common yeast genera isolated from wines were *Pichia* (5 species), *Saccharomyces* (1 species) and *Zygosaccharomyces* (2 species). Less frequent isolated genera were *Candida* (2 species), and only one species from genus *Cyberlindnera*, *Kregervanrija*, *Meyerozyma*, *Rhodotorula*, *Wickerhamomyces*, and *Torulaspora*. *Pichia* was the most common genus isolated from wine samples with 42 % representation. The second most common genus was *Saccharomyces* (34 %) followed by *Zygosaccharomyces* (11 %) and *Candida* (5 %). Other six identified genera with less than 5 % representation. Figure 1 shows the percentage representation of each genus of yeasts isolated from wine samples (total 285 isolates). According to Loureiro and Malfeito-Fereira [1] and Suárez *et al.* [4] wine can contain some

important wine spoiling yeast genera such as *Brettanomyces, Zygosaccharomyces, Pichia* and *Candida*. Authors also consider that *Brettanomyces bruxellensis, Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* are the most common spoilage yeast [1]. However, this last species (*S. cerevisiae*) appears to be more dangerous than other spoilage yeasts, as some strains isolated from dry white wines seem to be more potential spoilage yeast than *Z. bailii* due to its sorbic acid and sulphite tolerance at high ethanol levels [10]. According to Kunkee and Bisson [11], other spoilage and contaminant yeast species in wine can be divided into the following groups: (a) fermenting yeast *Saccharomyces cerevisiae* able to referment sweet bottled wines; (b) osmophilic yeast *Zygosaccharomyces bailii*; (c) aerobic film-forming yeasts (*Pichia* and *Candida*); (d) producer of ethyl phenols in the wine *Brettanomyces bruxellensis*. It has also been reported that *Brettanomyces bruxellensis* can form biogenic amines [12] that can lead to undesirable physiological effects in sensitive humans. *Brettanomyces* species need special selective cultivation media for cultivation, because of their slow growth and reduced presence in wine [11]. Some yeasts, e.g. *Candida* and *Pichia* are capable of forming films on the surface of wine exposed to oxygen. Off-odours, including acetic acid, ethyl acetate and acetaldehyde are also associated with their growth [13]. In our study identified two *Candida* species (*Candida inconspicua* and *C. sake*) from 7 different wine varieties, 5 red and 2 white. *Candida inconspicua* (6 %) found mainly in wines from Slovakia, Czech Republic and Spain. *Candida sake* (1 %) was found in red wine (Cabernet Sauvignon) from Italy. *Candida* species are able to produce sulphur compounds into the wine and these two species belongs to contaminant species [14-16]. Le Rouxet et al., [17] reported the presence of *Candida inconspicua* on grapes from Western Cape. Minervini *et al.* [18] found *C. inconspicua* to be a significant component of the yeast community in various Italian cheeses, in particular buffalo mozzarella. Suzzi *et al.* [19] reported the frequent isolation of the species from Manteca, a buttery whey cheese produced in southern Italy. According to Jolly *et al.* [20] *Candida* spp. are found predominantly on grapes and in freshly processed must. Lower numbers are found on winery equipment. In a study of Parish and Carroll [21], *Candida sake* was isolated from grape berries (variety Muscadine). We isolate and then identified both *Candida* species from wine samples, so these species can survive alcoholic fermentation and grow in wine. Some other *Candida* species were isolated in our study, but we present them as teleomorph. These were *Kregervanrija fluxuum* (anamorph *Candida vini*), *Pichia membranifaciens* (anamorph *Candida valida*), *Pichia occidentalis* (anamorph *Candida sorbosa*) and *Wickerhamomyces anomalus* (anamorph *Candida pelliculosa*, ex *Pichia anomala*) [22]. We identified *Kregervanrija fluxuum* (3 %) in 4 wine samples from Slovakia and in one sample from Hungary. The presence of *K. fluxuum* on grape berries was reported by Le Rouxet *et al.* [17] and in wine must by Combina *et al.* [23]. We also detected *Meyerozyma guilliermondii* (3 %) in four wine samples (2 grape varieties). *Pichia* species are more common in must and in wine as on the surface of grape berries. We identified five different *Pichia* species. The most common was *Pichia manshurica* (29 %, 29 white and 12 red wine samples) and *P. membranifacies* (13 %, 8 white and 10 red wine samples). Interestingly, *P. manshurica* was detected in 29 wine samples from Spain (total no. 32). *Pichia occidentalis, P. fermentans* and *P. norvegensis* were very rare (only 1 isolate from each species). These species were found in wines from different countries: *Pichia occidentalis* (1 %) (Welschriesling, Czech Republic), *Pichia fermentans* (1 %) (Cabernet Sauvignon, Italy) and *Pichia norvegensis* (1 %) (Blue Portugal, Slovakia). The species able to grow abundantly in wine, with aerobic and weakly fermentative metabolism are also known as film forming yeast (*Pichia membranifaciens, P. anomala* and *Candida* spp.). Given their oxidative metabolism and high growth rate, at winery temperature, they rapidly colonize surfaces contaminated with wine residues, being regarded as indicators of hygiene and of the stringency in avoiding wine contact with air [1]. *Pichia manshurica* and *Pichia membranifaciens* can produce volatile phenols in the wine, like similarly to *Brettanomyces* species [6]. *Wickerhamomyces anomalus* previously known as *Pichia anomala* and *Hansenula anomala* (anamorph *Candida pelliculosa*) is a very important yeast species with ability to produce proteinaceous mycocins (also known as killer toxins). The mycocin of *Wickerhamomyces anomalus* yeast is exo-ß-1.3

glucanase identified by MALDI-TOF/TOF with different antimicrobial activity against pathogenic *Candida* species [22, 24]. Satora *et al.* [25] used a mixed fermentation of *Wickerhamomyces anomalus* with *Saccharomyces cerevisiae* in fermentation of apple wines with positive results in chemical composition and sensory features. We isolated *W. anomalus* from two Spanish white wines and one red wine from Czech Republic. Other yeast with killer toxin activity was *Pichia membranifaciens* [26], *Saccharomyces cerevisiae* [27], *Zygosaccharomyces bailii* [28, 29], *Aureobasidium pullulans* [24] and *Metschnikowia pulcherrima* [30]. In our study genus *Zygosaccharomyces* was represented by two identified species: *Z. bailii* and *Z. florentinus*. *Z. bailii* was identified in 12 different wines and in 16 samples (11 %, 14 white and 2 red wines). According to Loureiro and Malfeito-Ferreira [1] *Zygosaccharomyces* spp. are considered to be winery contaminants and are especially a problem in wineries producing sweet and sparkling wines. Most of the literature recognizes these yeasts as spoilage organisms producing high quantities of acetic acid. Regueiro *et al.* [31] reported the presence of *Z. bailii* in red and white wines from Spain. We also identified *Z. bailii* from Spanish wines, especially found in Gruner Veltliner (4 samples). *Z. bailii* was also found in Tokay wines. These wines are semi-sweet and sweet with high content of residual sugars, e.g. Lipovina (19.3 g/L), "Samorodné" (Lipovina x Furmint) (64.3 g/L), Yellow Muscat (40.7 g/L) Tokay selection 3-tubs (94 g/L), Tokay selection 5-tubs (135.5 g/L) and Tokay selection 6-tubs (175.5 g/L). *Z. bailii* was detected also in wines with low residual content e.g. Furmint (2.4 g/L), Gruner Veltliner (1-1.3 g/L), or Blue Frankish (0.8 g/L). The second species *Z. florentinus* was detected only in two wine samples: Cabernet Sauvingon (Italy) and Pinot Blanc (Spain). Longo *et al.* [32] detected *Z. florentinus* in grape must varieties Abarino, Godello (white) and Mencia (red) from Spain [20]. Species as *Torulaspora delbrueckii* and *Cyberlindnera jadinii* (ex *Pichia jadinii*) was isolated from one wine sample. These two yeasts were identified in wines from Italy, *T. delbrueckii* (1 %) (Chardonnay) and *C. jadinii* (1 %) (Traminer). *Rhodotorula mucilaginosa* (3 %) was found in four different wine samples (two from Spain and two from Czech Republic). *Rhodotorula* spp. (*Rh. mucilaginosa, Rh. glutinis, Rh. minuta*) are widespread adventitious contaminants, but are not regarded as wine spoilers. Mainly they can be found on grapes, in fermenting must, and rarely in wine. They are common contaminants, but have little or no ability to grow in or spoil wines (obligate aerobes) [1]. Table 1 shows the list of yeast species identified by MALDI-TOF, number of isolates, grape varieties and country of origin of wine samples. *Saccharomyces cerevisiae* was identified in 34 wine samples (17 white and 17 red wines, 24 %). Presence of *S. cerevisiae* in wine samples divided by countries: 10 (CZ), 12 (SK), 6 (ESP), 4 (ITA) and 2 (HU). Comparison of *S. cerevisiae* with *Pichia manshurica*, detected in next wine samples: 3 (CZ), 11 (SK), 21 (ESP), 3 (ITA) and 3 (HU). *P. manshurica* with *S. cerevisiae* was found in two (similar?) wine sample from Slovakia (Gruner Veltliner and Blue Frankish), Spain (Pinot Blanc and Gruner Veltliner) and only in one same sample from Czech Republic (Cabernet Moravia), Hungary (Blue Portugal) and Italy (Traminer). Saez *et al.* [6] reported the presence of *P. manshurica* in spoiled wines (red and white). Synonym for *P. manshurica* is *P. galeiformis* and the incidence in wines and on sour rot damaged grapes was confirmed by several authors [6, 33-35].

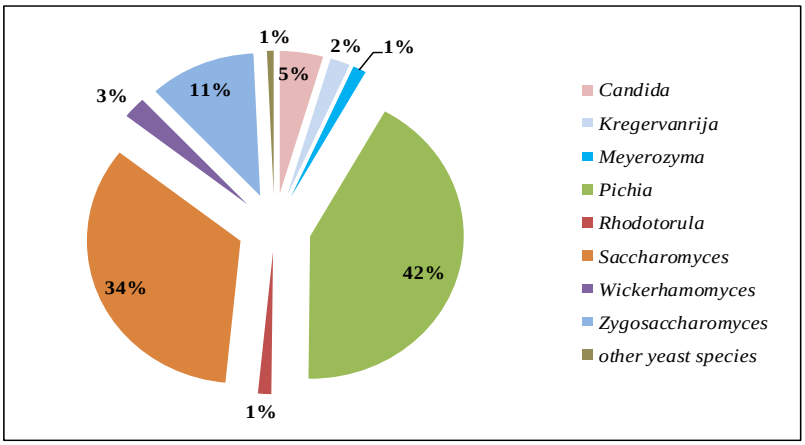

Figure 1. Percentage of genera of yeasts

Table 1. List of identified yeast species by MALDI-TOF MS

Yeast species	Grape variety	Wine	Country	No. of isolates
Candida inconspicua	Cabernet Moravia	red	CZ, SK	4
	Cabernet Sauvignon	red	CZ	1
	Cuvee	red	SK	1
	Blue Frankish	red	SK	1
	Macabeo	white	ESP	1
	Pinot Blanc	white	SK	1
	Zweigeltrebe	red	CZ	3
Candida sake	Cabernet Sauvignon	red	ITA	1
Cyberlindnera jadinii	Traminer	white	ITA	1
Kregervanrija fluxuum	Blue Frankish	red	SK	1
	Irsai Oliver	white	SK	1
	Blue Portugal	red	HU	1
	Traminer	white	SK	3
Meyerozyma guilliermondii	Blue Portugal	red	CZ, SK	3
	Muller Thurgau	white	HU	1
Pichia fermentans	Cabernet Sauvignon	red	ITA	1
Pichia manshurica	Cabernet Moravia	red	CZ	3
	Cabernet Sauvignon	red	ESP	1
	Cuvee	red	SK	1
	Blue Frankish	red	SK	8
	Furmint	white	SK	1
	Chardonnay	white	CZ, ESP, ITA	10
	Merlot	red	ITA	3
	Blue Portugal	red	SK, HU	7
	Muller Thurgau	white	HU, ESP	15
	Yellow Muscat	white	SK	1
	Welschriesling	white	ESP	7
	Pinot Blanc	white	ESP	1
	Sauvignon blanc	white	ESP	3
	Tokay selection 3-tubs	white	SK	1
	Traminer	white	ITA	1
	Gruner Veltliner	white	SK, CZ, ITA	20

Table 1. List of identified yeast species by MALDI-TOF MS (continued)

Yeast species	Grape variety	Wine	Country	No. of isolates
Pichia membranifaciens	Alibernet	red	CZ	2
	Cabernet Moravia	red	CZ, SK	1
	Cabernet Sauvignon	red	SK	4
	Cuvee	red	SK	8
	Blue Frankish	red	CZ, SK	1
	Macabeo	white	ESP	1
	Blue Portugal	red	CZ	8
	Muller Thurgau	white	HU, ESP	2
	Welschriesling	white	ESP	6
	Gruner Veltliner	white	ESP	1
Pichia norvegensis	Blue Portugal	red	SK	1
Pichia occidentalis	Welschriesling	white	CZ	1
Rhodotorula mucilaginosa	Dornfelder	red	CZ	1
	Muller Thurgau	white	ESP	1
	Welschriesling	white	ESP	1
	Zweigeltrebe	red	CZ	1
Saccharomyces cerevisiae	Alibernet	red	CZ	2
	Cabernet Moravia	red	CZ, SK	5
	Cabernet Sauvignon	red	CZ, ITA	8
	Dornfelder	red	CZ	2
	Blue Frankish	red	CZ, SK	5
	Merlot	red	ITA	3
	Blue Portugal	red	CZ, HU	9
	Muller Thurgau	white	SK	4
	Moravian Muscat	white	SK	3
	Welschriesling	white	SK	4
	Pinot Blanc	white	CZ, SK, ESP	4
	Pinot Noir	red	CZ	1
	Silvaner	white	SK	1
	Saint Laurent	red	CZ, SK	5
	Traminer	white	ESP, ITA	23
	Gruner Veltliner	white	SK, ESP	10
	Zenit	white	HU	1
	Zweigeltrebe	red	CZ	7
Torulaspora delbrueckii	Chardonnay	white	ITA	1

Table 1. List of identified yeast species by MALDI-TOF MS (continued)

Wickerhamomyces anomalus	Muller Thurgau	white	ESP	1
	Pinot Noir	red	CZ	1
	Traminer	white	ESP	5
Zygosaccharomyces bailii	Blue Frankish	red	SK	2
	Furmint	white	SK	1
	Chardonnay	white	ESP, ITA	4
	Lipovina	white	SK	2
	Blue Portugal	red	HU	1
	Yellow Muscat	white	SK	1
	Rheinriesling	white	CZ	2
	Samorodné	white	SK	1
	Tokay selection 3-tubs	white	SK	1
	Tokay selection 5-tubs	white	SK	1
	Tokay selection 6-tubs	white	SK	2
	Gruner Veltliner	white	ESP	12
Zygosaccharomyces florentinus	Cabernet Sauvignon	red	ITA	1
	Pinot Blanc	white	ESP	1

SK: Slovakia; **CZ:** Czech Republic; **HU:** Hungary; **ESP:** Spain; **ITA:** Ital

4. Conclusions

We identified by MALDI-TOF MS 16 different yeast species belonging to 10 genera, namely: *Candida* (5 %), *Cyberlindnera* (< 1 %), *Kregervanrija* (2 %), *Meyerozyma* (1 %), *Pichia* (42 %), *Rhodotorula* (1 %), *Saccharomyces* (34 %), *Wickerhamomyces* (3 %), *Zygosaccharomyces* (11 %) and *Torulaspora* (< 1 %). *Pichia manshurica* (29 %), *P. membranifaciens* (13 %), *S. cerevisiae* (24 %) and *Z. bailii* (11 %) were the most common species in wine samples. According to different authors the most dangerous species with negative impact on quality of wine are *Zygosaccharomyces*, *Pichia*, *Candida* and *Saccharomyces*. All identified species belongs to spoilage yeasts besides *Rhodotorula* (it's a common contaminant but with no ability to spoil wines).

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