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CHARACTERIZATION OF YEAST AND MOULD BIOTA OF BOTRYTIZED GRAPES IN TOKAJ WINE REGION IN THE YEARS 2000 AND 2001

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The most important base material of the famous wine Tokaji Aszú is the noble rotted grapes attacked by *Botrytis cinerea* under special conditions. The objective of this study was to determine the quantitative and qualitative composition of the yeast and mould biota present on the surface of aszú-berries in the year of 2000 and 2001, and to compare these findings with the observations made in previous vintages. The studied years represented extremely different conditions for the noble rot, so the effect of the vintage on the quantitative and qualitative composition of the microflora was more pronounced than in the earlier years. The excellent year of 2000 resulted in yeast and mould counts (mean logarithmic values of 4.47 and 4.72) significantly lower than found in the extremely poor vintage of 2001 (mean values of 6.58 for yeasts and 7.10 for total moulds). The place of sampling (vineyard or winery) had less impact on the quantitative composition of the microbiota than found in previous, less extreme years. The results of qualitative analysis, however, confirmed that the taxonomic composition of the yeast biota depends on the place of sampling, showing that the storage conditions of aszú grapes before vinification should be studied and optimized.

Keywords: *Botrytis*, noble rot, Tokaji, yeast

Tokaji Aszú, one of the oldest botrytized wines of the world, is produced from noble rotted grapes, called “aszú” berries. Formation of noble rot caused by the facultative parasitic mould *Botrytis cinerea* is frequently supported by the special climatic conditions, soil conditions and grape-varieties of Tokaj-region.

Noble rot is a combination of two strictly related processes: the special metabolic activity of *Botrytis* invading the grapes in full maturity and the physical concentration of the juice content of the berry due to evaporation. Berries lose water through the skin disrupted by *Botrytis* and since the vascular connection with the vine becomes depleted at the final state of maturity, the water cannot be replaced from the vine through the stem. Therefore, sugar and total soluble material content will be highly concentrated. As a common result of the enzymatic activity of *Botrytis* and the physical process of concentration, the grapes undergo complex chemical modifications, which have been well studied (reviewed by DITTRICH, 1987; DONECHE, 1993; JACKSON, 1994).

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The growth of the saprophytic micro-organisms such as other mould strains (e.g. *Penicillium, Aspergillus*) and yeasts is supported by digestion and mechanical disruption of the berry skin by *Botrytis cinerea*. The presence of saprophytic moulds may lead to the formation of unpleasant flavour and smell. Different yeast species also invade the disrupted berry skin and they may have either positive or negative impact on the quality of aszú wine during the special vinification process. One of the critical steps of aszú making is the alcoholic fermentation after maceration and extraction of aszú-berries with wine or fermenting must. In most of the wineries the fermentation has been actually uncontrolled relying on the indigenous yeast flora. Because of the special chemical composition of the alcoholic base wine gained after extraction of the noble rotted grapes, the fermentation of aszú wines has to face a lot of difficulties (MAGYAR, 1996; MAGYAR et al., 1999), so it might be either prolonged, stuck or might result in a sugar content lower then expected. Variable and often unpredictable occurrence of these problems depends on the taxonomic nature and physiological state of indigenous yeasts. Microbiota of botrytized wines other than Tokaji have been studied long ago (DOMERCQ, 1957; LE ROUX et al., 1973; DITTRICH, 1987) and the yeast flora in the Slovakian part of the historical Tokaj region was also investigated (MINARIK, 1963), but research data about the Hungarian Tokaj Wine District have been published only in recent years (MAGYAR, 1996; KALMÁR et al., 1999; MAGYAR et al., 1999; SIPICZKI et al., 2001; NAUMOV et al., 2002). In former studies we investigated the composition of the microbiota present on the surface of the aszú grapes produced in 1998 and 1999 (BENE & MAGYAR, 2002). As an extension of this work, we continued our investigations in the vintages of 2000 and 2001. The aim of this paper is to present the more up-to-date results in comparison with the earlier findings.

In the study, we examined the surface of berries taken from the vine-stock and the winery, paying attention to the change of microbiota as a function of origin and year of the botrytized grapes.

1. Materials and methods

One part of the aszú berry samples was taken aseptically from the vineyards, directly from the vine stocks. Another part of the samples was collected from the storage places of various winemaking companies after manual picking, sorting, transporting and storage.

Cells attached to the surface of aszú-berries were washed into sterile water with shaking, then after diluting they were spread-plated on different selective media. Total yeast and mould count was determined in Dichloran-Rose-Bengal-Chloramphenicol DRBC agar (Merck, Germany) and Dichloran-glycerine DG18 agar (Merck, Germany). The dichloran-content restricts the colony size to several moulds. The detection limit of the method was about 10^2 CFU g^{-1} berry.

The characteristic yeast colonies were isolated from several dilution levels, purified by serial streaking, investigated under microscope then identified according to

KURTZMANN and FELL (1998), and as detailed elsewhere (BENE & MAGYAR, 2002). All isolates were identified to the level of genera and most of them to the level of species. Moulds were identified to the level of genera according to morphological characteristics.

2. Results

2.1. Quantitative composition of micro-organisms on the surface of Aszú-berries

Climatic conditions in the two years considerably differed from the aspect of noble rot. While year 2000 offered extremely good conditions for producing high quality aszú grapes, the rainy autumn in 2001 provided an intensive growth of *Botrytis*, producing low quantity and lower quality noble rotted berries.

Total yeast and mould counts of the individual grape samples analysed are presented in Fig. 1 and Fig. 2. Mean values of the measured populations with statistical parameters are summarised in Table 1, where the effect of sampling place (winery or vineyard) can be evaluated.

Table 1. Comparison of the mean values of cell counts (log CFU g^{-1}) detected on the surface of aszú grapes taken from different sampling places (vineyard or winery) in two years

Lg N/g		2000			2001		
		Place of sampling			Place of sampling		
		vineyard	winery	difference	vineyard	winery	difference
Total yeast	mean	4.474	5.071	–0.597	6.581	6.115	0.466
	Variance	0.7819	1.9058		0.7914	1.3473	
	n	4	9		10	12	
	t	0.78		P = 0.4485	1.04		P = 0.3113
	$t_{5\%}$	2.20			2.08		
Botrytis	mean	4.535	3.978	0.557	7.012	6.274	0.738*
	Variance	3.1758	5.6951		0.3376	0.9377	
	n	4	9		10	12	
	t	–0.4144		P = 0.6865	2.1095		P = 0.0477
	$t_{5\%}$	2.20			2.08		
Other mould	mean	3.687	1.735	1.952	4.921	5.632	–0.711
	variance	7.6346	4.5531		3.9039	1.3830	
	n	4	9		10	12	
	t	–1.3981		P = 0.1896	–1.0460		P = 0.3080
	$t_{5\%}$	2.20			2.08		

n: number of samples.
t: calculated *t*-value.
$t_{5\%}$: critical *t*-value at P = 0.05.
P: significance level of *t*-test. Difference between the mean values is considered significant if P < 0.05 (*) and highly significant if P < 0.01 (**).

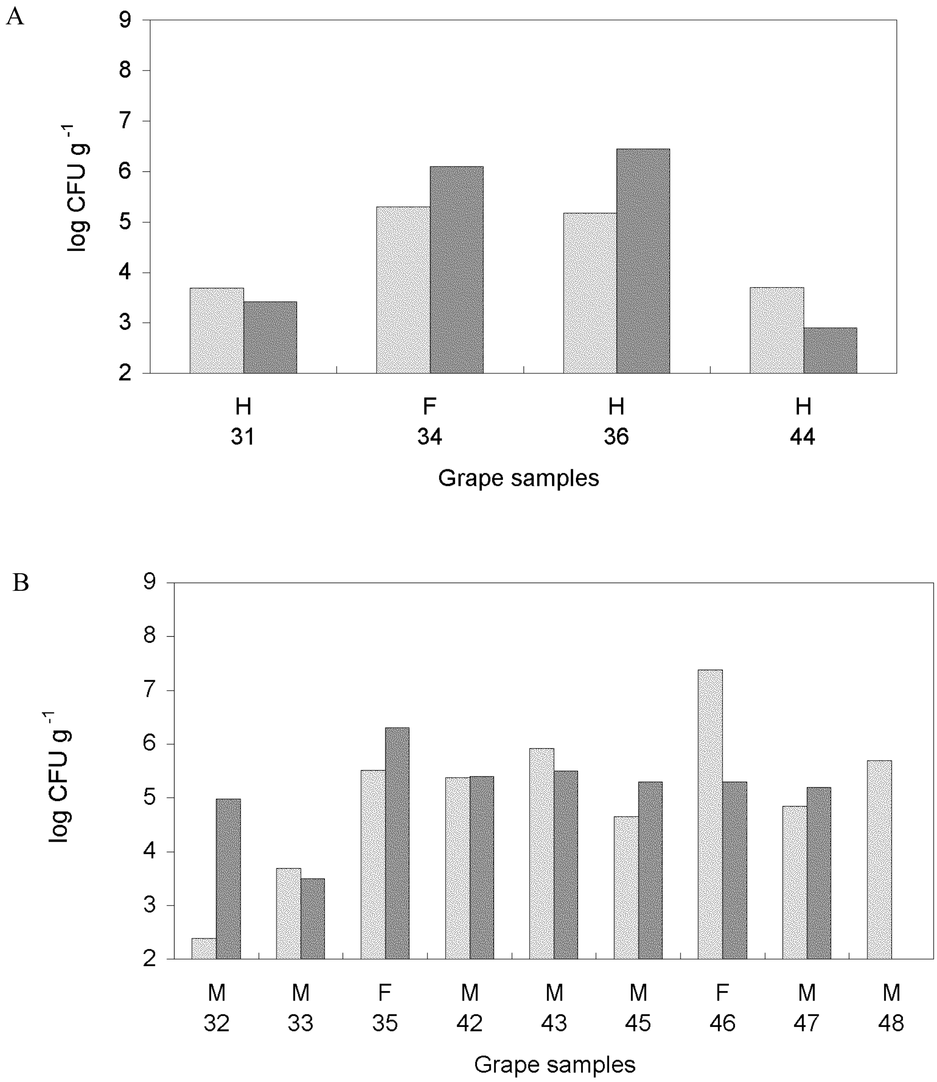

Fig. 1. Yeast and mould counts on the surface of noble rotted grapes taken from the vine before harvest (A) and from the winery after storage (B) in the year 2000 (Grape varieties: H: Hárslevelű; F: Furmint; M: mixed). ▒: Total yeast; ■: total moulds

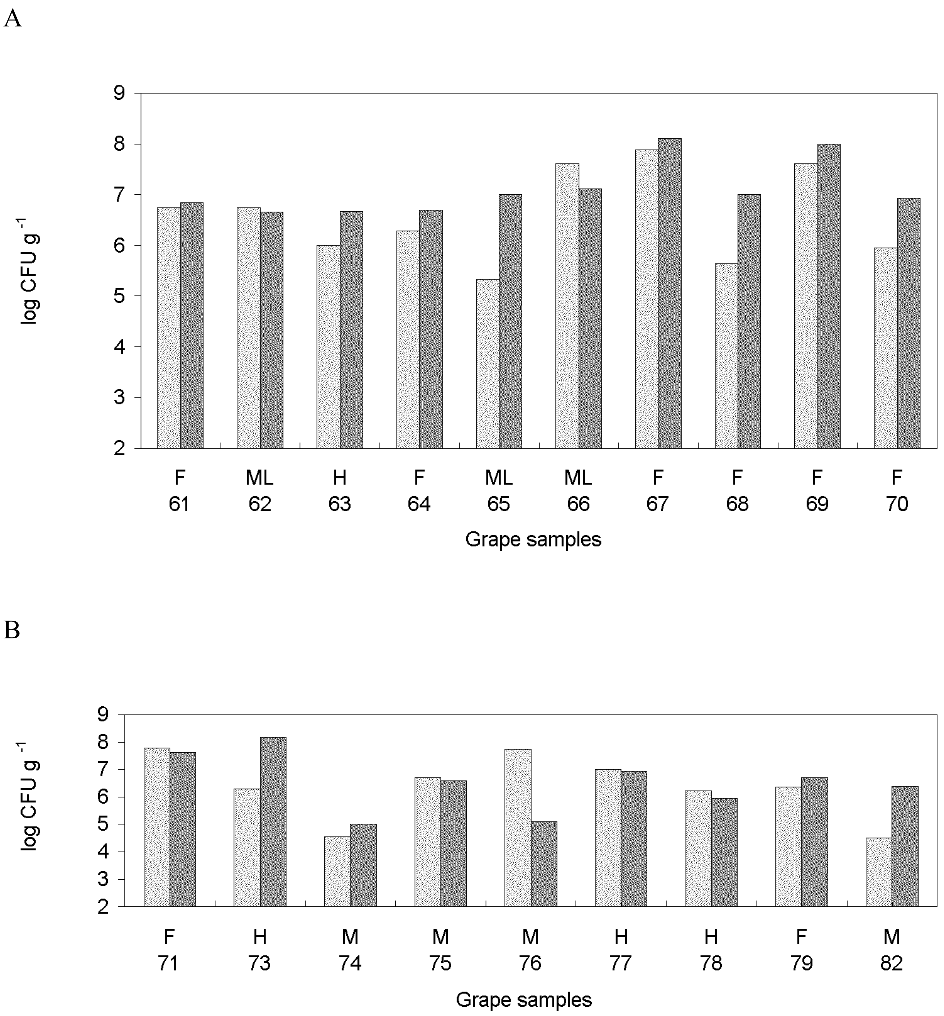

Fig. 2. Yeast and mould counts on the surface of noble rotted grapes taken from the vine before harvest (A) and from the winery after storage (B) in the year 2001 (Grape varieties: H: Hárslevelű; F: Furmint; ML: Muscat lunel; M: mixed). ▒: Total yeast; ■: total moulds

2.1.1. Moulds. Variability of the total mould count including *Botrytis* and other species is shown in Fig. 1 and Fig. 2. It has to be noted that, as a consequence of the surface washing method, the data actually mean conidia-counts on the surface of the examined berries, which are not necessarily proportional to the mass of mycelium spread through skin.

Botrytis conidia (Table 1) were present in higher density in the samples of year 2001 than in 2000 (mean logarithmic values in the vineyard are 4.5 and 7.0, respectively). This must be a consequence of the vintage, because the drier year 2000 supported mycelial growth of *Botrytis*, while conidium formation was suppressed. During storage of the grape, the low conidia number did not show significant changes in this year.

In the rainy year 2001, the formation and increase of conidia had a more noticeable degree. In the case of *Botrytis* conidia-number, the difference between the mean values of vineyards and wineries was significant (Table 1). From this result, it is clear that there was a reduction during the storage.

Besides *Botrytis,* other mould species like *Penicillium, Aspergillus,* were commonly found in widely varying populations, but the average conidia number was 1–2 log cycles lower than that of *Botrytis*. In 2001, their count was measured between 10^3–10^6 CFU g^{-1} (data not shown separately) and, in contrast to the *Botrytis* conidia, it did not decrease during the storage. The relatively high count of saprophytic moulds may adversely influence the quality of aszú grape. This directs attention to the importance of storage conditions, especially in years when the humidity is high during the formation of noble rotted berries.

2.1.2. Yeasts. Similarly to mould count, in the excellent year 2000 the average yeast count on the aszú grapes was much lower than in 2001 (mean logarithmic values in the vineyard were 4.47 and 6.58, respectively). The different levels show that the lower quality botrytized grapes provide more favourable parameters to the growth of yeasts than the high quality grapes. This can be explained with the lower sugar content and with the stronger infection of *Botrytis*, because the more disrupted berry skin gives more possibility to the concomitant microbiota (yeasts and other moulds) to grow.

Within one year, the quantitative differences between samples from the vineyard and those from the winery were not significant in this study (Table 1). In earlier studies, we measured significant reduction of the total yeast count during storage of the aszú grapes in a good year, and significant increase in a poor quality vintage (BENE & MAGYAR, 2002). We explained these tendencies by the higher sugar content of the higher quality aszú grapes. The extremely good and extremely bad years studied in the present work did not confirm this conclusion, showing that the factors influencing the quantitative changes of the yeast biota are more complex than the high sugar content itself.

There was no noticeable correlation between yeast counts and grape varieties, although this question should be further investigated in larger number of samples.

2.2. Taxonomic composition of the yeast biota of aszú grapes

During this study, we isolated 68 yeast-strains, which were identified according to KURTZMANN and FELL (1998). Tables 2 and 3 show the qualitative composition of the yeast-biota in 2000 and 2001, respectively. The identification was not successful in some cases, because several strains died off before the tests were completed.

Table 2. Taxonomic composition of the yeast biota detected on the surface of aszú-grapes taken from the vine or from the winery in 2000

Place of sampling	Grape-variety	Code of samples	Cell count of the dominant yeast-strain (log CFU g^{-1})	Dominant yeast genera or species	Other yeast-species isolated
Vine-stock	Hárslevelű	31	3.47	Non-identified	
	Furmint	34	5.39	*Candida pulcherrima*	*Candida stellata*, *Rhodotorula* sp.
	Hárslevelű	36	5.00	*Hanseniaspora uvarum*	*Sporidiobolus* sp., *Candida sp.*, *Candida dendrica*
	Hárslevelű	44	3.57	*Candida stellata*	*Candida pulcherrima*
Winery	Mixed	32	2.48	*Cryptococcus* sp.	
	Mixed	33	3.47	Non-identified	
	Furmint	35	5.18	*Candida pulcherrima*	*Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Candida* sp.
	Mixed	42	4.98	*Sporidiobolus* sp.	*Candida stellata*, *Candida paludigena*, *Candida catenulata*
	Mixed	43	5.84	*Candida sake*	*Candida stellata*, *Filobasidium capsuligenum*
	Mixed	45	4.28	*Candida stellata*	
	Furmint	46	7.00	*Candida stellata*	
	Mixed	47	4.97	*Candida catenulata*	

On the aszú-berries taken from the vineyard directly, the most dominant species was *Candida pulcherrima* (teleomorphic state: *Metschnikowia pulcherrima*) in both years followed by *Hanseniaspora* and some basidiomycetes-related aerobic species in 2001, and by *Candida stellata* in the excellent year 2000. Similar observations were made during our previous studies in 1998 and 1999 (BENE & MAGYAR, 2002).

The taxonomic composition of the yeast biota on the samples taken from wineries was different comparing with the aszú grapes being on the vine stock. The change was more pronounced in 2000, when the sugar-tolerant *Candida stellata* became dominant in most of the samples stored in the wineries. In 2001 dominance of *Candida pulcherrima* and *Hanseniaspora* species remained or even increased during storage.

Dominance of *Candida stellata* on the noble rotted grapes was reported in the wine districts of Gironde (DOMERQ, 1957) and was often associated with the *Botrytis* infection (GANDINI, 1973; LE ROUX et al., 1973; FLEET & HEARD, 1993). In this study dominance of *C. stellata* was typical only in the good year, and mainly in samples taken from the wineries, not in the vineyard. These results generally confirm our earlier observations (BENE & MAGYAR, 2002).

Table 3. Taxonomic composition of the yeast-biota detected on the surface of aszú-grapes taken from the vine or from the winery in 2001

Place of sampling	Grape-variety	Code of samples	Cell count of the dominant yeast-strain (log CFU g^{-1})	Dominant yeast genera or species	Other yeast-species isolated
Vine-stock	Furmint	61	6.54	*Candida pulcherrima*	*H'spora guilliermondii*
	Muscat	62	6.54	*Hanseniaspora uvarum*	*Candida stellata*
	Hárslevelű	63	6.00	*Trichosporon pullulans*	–
	Furmint	64	6.02	*Candida stellata*	*Hanseniaspora vineae*
	Muscat	65	5.20	*Sporidiobolus pararoseus*	*Candida pulcherrima*
	Muscat	66	7.45	*Candida catenulata*	*Candida pulcherrima, Candida stellata*
	Furmint	68	5.56	Non-identified	*Sporidiobolus pararoseus, Trichosporon pullulans*
	Furmint	69	7.57	*Brettanomyces nanus*	*Sporidiobolus pararoseus*
	Furmint	70	5.65	*Kluyveromyces lactis* var. *drosophilarum*	*Sporidiobolus pararoseus*
Winery	Furmint	71	7.77	*Hanseniaspora guilliermondii*	*Candida pulcherrima, Dekkera bruxellensis*
	Hárslevelű	72	5.69	*Candida pulcherrima*	
	Hárslevelű	73	7.02	*Candida pulcherrima*	*Metschnikowia pulcherrima*
	Mixed	74	4.54	*Candida bombicola*	
	Mixed	75	7.39	*Dekkera bruxellensis*	*Candida catenulata*
	Mixed	76	7.72	*Candida pulcherrima*	
	Hárslevelű	77	6.78	*Sporidiobolus pararoseus*	*Hanseniaspora osmophila,* Non-identified
	Hárslevelű	78	6.20	*Candida stellata*	
	Furmint	79	5.95	*Candida pulcherrima*	*Kluyveromyces lactis* var. *drosophilarum, H'spora guilliermondii,* Non-identified
	Furmint	80	6.04	*Hanseniaspora guilliermondii*	*Candida pulcherrima*
	Mixed	81	4.39	*Hanseniaspora guilliermondii*	
	Mixed	82	4.50	Non-identified	

In contrast to other authors (NAUMOV et al., 2002), we could not isolate *Saccharomyces* strains from the surface of aszú grapes, irrespective of the place of sampling or year.

3. Conclusions

Mould and yeast biota of the noble rotted grapes in Tokaj region is quantitatively and qualitatively influenced by the extremes of vintages, but between typical years, only slight differences can be found. From the research data of four vintages (1998, 1999 published elsewhere and 2000, 2001 presented here) we concluded that dry, high quality vintages provided low yeast populations with low mould conidia count.

In the vineyard, *Candida pulcherrima* proved to be the most typical dominant yeast of the aszú berries. During collecting, sorting, transporting and storing, microbiota of the noble rotted berries undergo considerable qualitative changes due to the high selective pressure of the special micro-ecological conditions, which typically leads to the prevalence of *Candida stellata* (the extremely low quality vintage of 2001 seems to be an exception). Diversity of the yeast species generally decreases during storage, particularly in the good years, but the total yeast population may either decrease or grow influencing the quality of the grapes. Further research is in progress to determine the impact of microbiota on the aszú grape quality, as well as to optimise the storage conditions of aszú berries.

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