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ASSESSMENT OF GRAPES INDIGENOUS MICROBIOME FROM "ȘTEFAN VODĂ" PROTECTED GEOGRAPHICAL INDICATION

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Abstract

The diversity of the yeast microflora significantly contributes to the chemical and sensorial characteristics of wine. Lately, the significance of microbiome of grape berries of different geographical origin and varieties, winemaking practices and climatic conditions has led to the suggestion of the microbial terroir. Under micro winery conditions were studied indigenous flora of Cabernet Sauvignon and Merlot grapes from "Ștefan Vodă" PGI. This study is reflecting that the dynamics of the alcoholic fermentation process presented several peculiarities: the spontaneous fermentation had a latency period of 4 days until the beginning of the active phase, and then presented difficulties in completion, especially at the end of the fermentation. Due to possible early and significant development of Kloeckera apiculata, spontaneous fermentation was accompanied by an increase in volatile acidity. The microscopy of studied samples allowed their visual evaluation and the preventive determination of some morphological characters of the microbiome. An efficient use of the grape microbiome would be the selection and subsequent multiplication to be used for fermentation (starter cultures).

Key words*: authenticity, grape microbiome, indigenous flora, wine.*

INTRODUCTION

Winemaking is characterized by a constant decrease of microbial diversity, both bacteria and yeasts. In the vineyard, yeast populations are low and compete with moulds. Most species are oxidative, so they do not, or very little, transform grape sugars into alcohol. This is why it can be found very few fermentation yeasts of the *Saccharomyces cerevisiae* type on grapes (Delteil, 2000; Pretorius, 2020). But in the fermenting must, we do not systematically find yeasts from the grapes, both in terms of present species and of strains deriving from them. At the same time, the cellar is characterized by an atmosphere loaded with both fermentative and oxidative yeasts (Poulard, 2008).

When the use of SO_2 in the must is low or zero, and the fermentation temperatures are in the range from 10 to 25˚C, the fermentation is generally carried out by several species of yeasts which intervene at different fermentation stages. So, apart from grapes microbiome, the cellar can also be a significant source of contamination (Beguin et al., 2003; Coarer M., 2008).

Spontaneous fermentation is ensured by indigenous flora which is not selected. According to Poulard studies (2008) several peculiarities are revealed. Immediately after settling, different species of apiculate and oxidative yeasts develop, producing very low amount of alcohol: *Kloeckera, Candida, Metschnikowia,* etc. Afterwards, a large number of different strains of *Saccharomyces cerevisiae* (between 10 and 20) take over, including only 1 or 2 are dominant either by their numerical importance (50%), or by their presence throughout the fermentation process (Poulard, 2008). Other strains of *Saccharomyces cerevisiae* may appear exceptionally depending on environmental conditions (particularly alcohol content), and always in insignificant proportions. Oxidative species can, however, intervene occasionally during various winemaking operations, notably involving a supply of oxygen in the musts: pumping over, aeration, etc. (Ribereau-Gayon et al., 2004).

The evolution of dominant strains has a direct impact on fermentation. These strains can sometimes persist for several years, but it is more common to record an annual renewal (Poulard, 2008).

Torulaspora delbrueckii yeasts produce specific aromatic profiles during fermentation, improve the organoleptic properties of wines

and it is recommended to use them together with *Saccharomyces strains*. The use of *Torulaspora delbrueckii* yeasts in winemaking is possible in combination with *Saccharomyces cerevisiae* strains, yeasts that ensure the completion of alcoholic fermentation in a reasonable time. Studies on the synergism of the interaction between these two species are relatively few, especially from the point of view of quantifying the effect of this interaction (Lonvaud, 2004; Beguin et al., 2008; Poulard, 2008; Liu et al., 2019). Another factor that is important to be taken in view is the influence of the nutritional environment on the development of these two different strains (Poulard, 2008).

Regarding the terroir, it is difficult to associate active microflora on the must in winemaking because of great diversity: geographical (no individual or population strictly associated to a region), temporal (no rigorous rule, except variability), taxonomic (no single species or genus), genetic (no single-strain fermentation) (Liu et al., 2019; Kazou M., 2023). On the other hand, the terroir, just like the cultivation practices and the climatic conditions of the vintage, undoubtedly has a more or less direct impact on the overall structure of the yeast microflora (Renouf et al., 2006; Liu et al., 2019; Oyuela Aguilar et al., 2020).

If spontaneous fermentations can often take place without any problem, in around 30% of cases we see difficult and sluggish fermentations. Unlike fermentation accidents, these situations are difficult to correct and have consequences on aroma profile involving more or less significant depreciation. The presence of lactic bacteria during these sluggish fermentations can also lead to undesirable effects. Maintaining significant populations of acetic bacteria until the start of alcoholic fermentation increases the phenomena of SO_2 recombination (Beguin et al., 2008).

Certainly, spontaneous fermentation is not responsible for all the quality problems that may arise, but it is carried out by a microbial consortium that is difficult to control. Poorly controlled, it leaves the way open to multiple complications. Spontaneous fermentation therefore constitutes, in all cases, a risk that must be carefully calculated and taken with full knowledge of the facts (Beguin et al., 2003).

It can be mentioned that yeast diversity does not rhyme with complexity, nor with diversity. On the other hand, a large number of fermentative strains can create problems in terms of fermentation. The struggle for nutrients between different strains may be the cause of sluggish fermentations (Coarer M., 2008).

Winemakers could not rely on the spontaneous development of indigenous yeasts to benefit from the positive effects because of the risk of uncompleted fermentation and inherent sensory deviations. In this context, an important task in the field of wine microbiology is the identification and selection of indigenous yeasts with valuable technological properties and easily adaptable to the environment, which ferment completely the carbohydrates from the must in order to obtain natural wines with high organoleptic qualities and to guarantee wines authenticity (Koralewski, 2010; Pretorius, 2020).

Therefore, there is a need to know the biochemical and physico-chemical characteristics of the wines produced in each winemaking center based on the selection of certain strains of local yeasts, the results of which could be used to guarantee the authenticity of wines with protected designations of origin or protected geographical indications.

MATERIALS AND METHODS

Researches were carried out within the Oenological Research Center of the Technical University of Moldova. The grape samples were harvested in October 2023 in Javgur village from Cimișlia district, which is part of a natural amphitheatre located on the ancient "Valul lui Traian de Sus", belonging to PGI "Ștefan Vodă".

Grapes of Cabernet Sauvignon and Merlot varieties were randomly collected from vineyards and transported immediately (4 hours on average) for microbiological and physico-chemical analyses. Subsequently, the harvested grapes were crushed and destemmed. Afterwards, the must was submitted to the maceration-fermentation process in micro winery conditions in 5.0 L glass container on indigenous microflora at a constant temperature

of 28 ± 1°C. The spontaneous fermentation of the must occurred starting with the 4th day after the grapes processing and lasted for 31 days until the complete fermentation of sugars. After the completion of the fermentation-maceration process, the must was pressed, the young red wine was directed to post fermentation and subjected to physico-chemical and microbiological analyses.

The physico-chemical analyses were performed in order to establish the basic composition (alcoholic strength, total acidity, volatile acidity, reducing sugar, pH), the polyphenolic composition (Folin-Ciocalteu index) and the chromatic characteristics (Color intensity, shade) in accordance with official International Organization of Vine and Wine (OIV) practices (OIV-MA-INT-00-2021, Compendium of International Methods of Wine and Must Analysis) and national Technical Regulation "Methods of analysis in the field of winemaking" (HG RM no. 708 of 20.09.2011).

The content of total phenolic substances was determined by the UV–VIS spectrophotometry method with the Folin-Ciocalteu reagent, gallic acid (Sigma-Aldrich) being used as a calibration substance. Prior to determination of phenolic compounds, the red wines were centrifuged at 8000 rpm for 15 minutes.

Chromatic indices were measured spectrophotometrically using quartz cuvettes with 1 mm optical path. The UV-VIS spectrophotometer was used to measure the absorption and transmission of light in the UV spectrum and the visibility of samples. The chromatic indices were calculated according to the formulas (OIV-MA-AS2-07B-2021, Compendium of International Methods of Wine and Must Analysis):

Colour intensity: $Ic = (A_{420} + A_{520} + A_{620})$;

Colour shade: $Nc = A_{420} / A_{520}$.

A_{420} – the absorbance value at 420 nm, characterizes the yellow component of the color; A_{520} – the absorbance value at 520 nm, characterizes the red component of the colour;

A_{620} – the absorbance value at 620 nm, characterizes the purple component of the colour.

The sensory analysis of the was carried out by a group of 12 tasters, which provided the description of the sensorial profile. Each descriptor was scored by points between 1 (least felt) and 10 (most felt) and then recorded in a special descriptive evaluation sheet (Regulation on the Evaluation Method of the Organoleptic Characteristics of Wine Products through Sensory Analysis).

The microbiological study (microscopy, yeast cells counting, seeding on different nutrient media, etc.) was carried out according to the national instructions of the microbiological control of wine production (IC MD 67-42582515-01-2010) and the OIV resolution (OENO-MICRO 08-370, version 2012). The results of the studied samples microbial load were compared with the limit values described in the normative documents (OIV-MA-INT-00-2021, OENO-MICRO 08-370).

In order to assess the microbiota of Merlot and Cabernet-Sauvignon grapes was used the method of washing water microscopy, which consisted in washing for 3 minutes 300 grames of grapes (the samples must contain both berries and portions of stems) in 500 mL distilled water so the microorganisms from the grape elements would pass into the water. Afterwards, the washing water was used for direct microscopy and seeding on media: Sabouraud 4% Dextrose Agar (SDA) medium (agar 18 g/L, dextrose 40 g/L, peptone 10 g/L) (Sigma-Aldrich); MRS Agar Vegitone medium (yeast extract 5 g/L, proteose peptone (vegetable) 10 g/L, dextrose 20 g/L and agar 15 g/L) (Sigma-Aldrich); Brettanomyces Agar Base (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, dextrose 10 g/L and agar 20 g/L) (HiMedia); Lauril Triptose Broth (tryptose 20 g/L, lactose 5 g/L, sodium chloride 5 g/L, potassium hydrogen phosphate 5.5 g/L) (HiMedia).

The inoculation was carried out on the surface of the sterilized media, melted and cooled by scarification or streaking, after which the media were placed in a thermostat at 30ºC. The samples are thermostated for 7 days, but periodically visual analysis of the media was performed to monitor the development and growth of microorganisms on the media.

In order to establish the purity of the isolated yeasts, microscopy of the grown cultures was performed. For this, the yeast strains were pre-incubated for 5 days on a nutrient medium at a temperature of 30°C. Samples were seeded on the following media: Sabouraud Dextrose Agar

(for the detection of pathogenic and non-pathogenic fungi), MRS Agar (for the detection of *Lactobacillus* lactic bacteria), Broth (non-selective detection of microorganisms), Brettanomyces Agar (for the detection of *Brettanomyces*).

After the completion of the spontaneous fermentation, a new seeding was performed on the MRS Agar, Brettanomyces Agar and Sabouraud Dextrose Agar medium and after a 5-day thermosetting, the visual analysis and microscopy of the cultures was performed. Depending on the results of the samples microscopy the microbiological state of the wine samples was established.

RESULTS AND DISCUSSIONS

The geographical location of the "Ștefan Vodă" PGI area is characterised by insufficient humidity and specific climatic conditions due to nearness of Black Sea. According to statistical data, the amount of precipitation varies from 450 mm to 550 mm. The presence of tertiary red minerals, rich in iron and micro-elements of the iron group explains the production of superior quality wines in this region.

Red grapes of Cabernet Sauvignon and Merlot varieties were taken from the vineyards of Javgur village, physical-chemical indices were recorded in Table 1.

Table 1. Physical-chemical indices of studied grapes

Physical-chemical indices	Cabernet Sauvignon	Merlot
Mass concentration of sugars, g/L	220 ± 1	234 ± 1
Mass concentration of titratable acids, g/L	6.5 ± 0,1	6.2 ± 0.1
Phenolic compounds technological potential, mg/L	3700 ± 15	3380 ± 15

The values of the content of phenolic compounds for Cabernet Sauvignon and Merlot varieties are characteristic for wines from the Southern region of Moldova, which stand out for a sum of active temperatures reaching an annual rate of 3200 to 3400°C, which ensures a full ripening of the grapes and highlight the wine-growing value of the region. Especially that the 2023 year in Republic of Moldova was characterized by an extremely high thermal regime and a significant deficit of precipitation in the July-October period (State Hydrometeorological Service from Republic of Moldova, 2023).

Nevertheless, from Table 1 it can be noticed that the content of phenolic compounds of the Cabernet Sauvignon variety is higher compared to that of Merlot, an aspect that is fully reflected in the specialized researches (Țardea C., 2007; Musteață et al., 2012; Tudose-Sandu-Ville et al., 2012) and foremost due to an increased technological reserve of phenolic compounds of Cabernet Sauvignon grapes.The dynamics of the alcoholic fermentation process of the studied musts presented several peculiarities: the spontaneous fermentation had a latency period of 4 days until the beginning of the active phase, and then presented difficulties in completion, especially at the end of the fermentation (Figure 1).

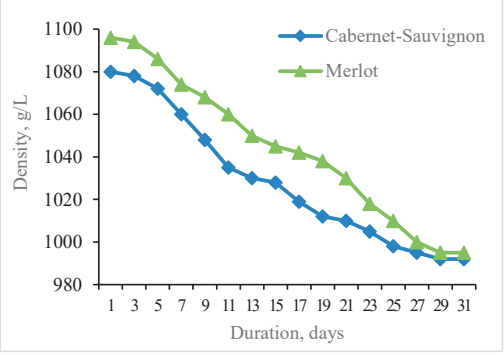


Figure 1. Kinetics of spontaneous fermentation of musts

The increasing concentration of alcohol, which accumulated in the must fermentation process, inhibited the activity of some yeast strains and slowed down the alcoholic fermentation process. Thus, spontaneous fermentation turned out to be less efficient: latency time - 4 days, total duration of alcoholic fermentation - 31 days, which once again proves the advantage of using starter cultures. After the completion of the alcoholic fermentation, the obtained wines were subjected to physical-chemical analysis, the results being presented in Table 2.

Table 2. Physical-chemical indices of studied wines

No	Physical-chemical indices	Cabernet Sauvignon	Merlot
1.	Concentration of alcohol, % vol	12.63 ± 0.1	13.45 ± 0.1
2.	Mass concentration of sugars, g/L	3.4 ± 0.1	4.5 ± 0.1
3.	Mass concentration of titratable acids, g/L	7.7 ± 0.1	7.6 ± 0.1
4.	Mass concentration of volatile acids, g/L	0.75 ± 0.05	0.68 ± 0.05
5.	pH	3.65 ± 0.01	3.37 ± 0.01
6.	Ic	18.6 ± 0.3	17.4 ± 0.3
7.	Nc	0.63 ± 0.02	0.653 ±0.02
8.	Organoleptic quality, points	7.80 ± 0.05	7.85 ± 0.05

According to the results presented in Table 2, it can be mentioned that the dry red wines obtained by spontaneous fermentation are characterized by a high concentration of alcohol.

The mass concentration of titratable acids in the wines obtained under micro winery conditions changed insignificantly, and the variation of the pH index values in the samples of dry wines obtained by spontaneous fermentation is within a limited range and constitutes 3.65 for Cabernet Sauvignon and 3.37 for Merlot.

The mass concentration of volatile acidity varies in both obtained wines (0.68-0.75 g/L), which can be explained by the development of different enzymatic reactions and latent fermentation conditions. These results could be explained by the early development of *Kloeckera apiculata* during spontaneous fermentation, which is accompanied by an increase in volatile acidity and ethyl acetate content (Poulard, 2008). Thus, especially due to the early and significant development of *Kloeckera apiculata*, spontaneous fermentation is accompanied by an increase in volatile acidity compared to induced fermentation.

Regarding the chromatic indices, it can be observed that Cabernet Sauvignon wine has a colour intensity with about 6.5% higher than in case of Merlot wine, which is specific for these varieties (Musteață et al., 2012; Tudose-Sandu-Ville et al., 2012).

On the other hand, the colour hue is only 1% bigger in the case of Merlot wine compared to Cabernet Sauvignon. The color hue, defined as ratio (A420/A520) showing the red color shift to orange hues is correlated with aging of the wine (Ribéreau-Gayon et al., 2006). Thus, the obtained results (Nc<1) are completely representative for young red wines, which are defined by the red colour of anthocyanidins (Musteață et al., 2012; Tudose-Sandu-Ville et al., 2012). This specificity grants them a better resistance and a higher color stability during the maturation and aging processes. Most often, spontaneous fermentations can take place without problems, but in about 30% of cases, difficult and prolonged finishing can take place (Coarer, 2008). The research carried out did not highlighted the diversity of the wines obtained by spontaneous fermentation complexity, the only measurable impact being the extension of the complete fermentation duration compared to selected yeasts. The microscopy of yeast preparations allowed their visual evaluation and the preventive determination of some morphological characters, such as: size, shape, grouping, as well as cell homogeneity. Following the microscopy of the washing water of Cabernet-Sauvignon and Merlot grapes, the presence of yeasts of the genus *Saccharomyces* and *Kloeckera apiculata*, of acetic bacteria in the form of bacillus, were detected in both samples.

Within three days, after further microscopy, it was found that the cells of the studied strains differed in shape and size, it was also established that the cells of the studied strains were eukaryotic and in budding state (Figure 2).

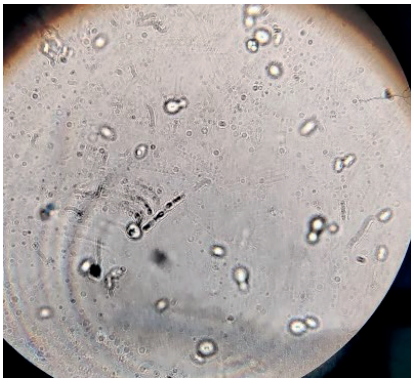
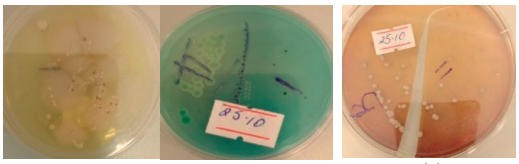


Figure 2. Microscopy of the washing water after 3 days

Seeding was performed on several types of media, and growth of different types of cultures was observed after visual analysis. In Figure 3 are represented the final results of the thermosetting of the media (Sabouraud SDA, Brettanomyces, Broth) and their visual analysis.



(a) (b) (c)

Figure 3. Results of seeding on different media:
(a) - Sabouraud SDA; (b) – Brettanomyces; (c) - Broth

Thus, it was found that on the Sabouraud SDA medium - from sample I (Cabernet Sauvignon) nothing developed and from sample II (Merlot) 3 different kinds of colonies have been evidenced:

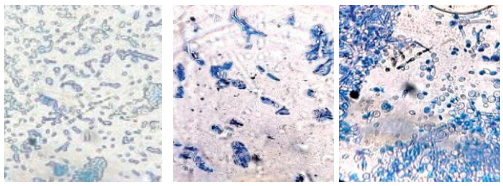
- colony 1 - red-brown, small, round, slightly convex, smooth, glossy;
- colony 2 - white, round, medium-sized, slightly convex, glossy;
- colony 3 - matte white color, with a large surface, irregular shape, flat.

On the Brettanomyces medium from both samples developed white-gray colonies of small size, convex and glossy. From the first sample, 4 colonies develop, and from the 2nd a much larger amount.

On the Broth medium from both samples developed a large number of medium-sized, round, glossy, slightly convex, grayish-white colonies.

Following the microscopy of the cultures developed on the media, the presence of the following microorganisms was found:

- on the Sabouraud SDA medium - the presence of yeasts from the genus *Saccharomyces cerevisiae*, *Kloeckera apiculata* and *Torulopsis* was detected (Figure 4 a).
- on the Bretanomyces agar medium - yeasts from the genus *Brettanomyces* were detected (Figure 4 b).
- on the Broth medium – presence of yeasts from *Saccharomyces* and *Brettanomyces* genus was found (Figure 4 c).



(a) (b) (c)

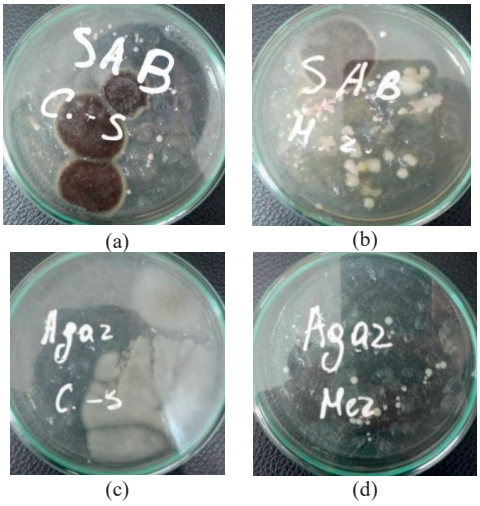
Figure 4. Results of seeding on different media:
(a) - Sabouraud SDA; (b) – Brettanomyces; (c) - Broth

After finalizing of the spontaneous alcoholic fermentation and settling of the wine, a new seeding was performed on the Agar and Sabouraud medium and after a 5-day thermosetting, the visual analysis and microscopy of the colonies was performed (Figure 5).

Following the visual analysis of both media, the development of two types of colonies was observed from both samples:

- the first type - grayish-white colonies, glossy, medium and small in size, round, with continuous, regular, slightly convex edges.
- the second type - white, matte, large, irregular, flat colonies.

In specialized literature (Kurtzman & Fell, 2000) is revealed that yeasts of the genus *Saccharomyces* have a round or ellipsoidal cell shape, the cells of the yeasts of the genus *Torulopsis* are spherical, and the lemon or cylindrical shape is characteristic of the cells of the genera *Hanseniaspora*, *Kloeckera*.



(a) (b) (c) (d)

Figure 5. Results of wine samples seeding
on different media: (a), (b) - Sabouraud SDA;
(c), (d) - Brettanomyces agar

Therefore, it was established that the studied yeasts do not form real mycelia, they all multiply vegetatively by multilateral budding and sexually by spores, which confirms that these strains belong to the genus *Saccharomyces*.

Thus, following the evaluation of the morphological, physiological and reproductive properties, it was found that the new yeast cultures isolated from the indigenous microflora are characterized by uniform yeast cells and are microbiologically viable.

CONCLUSIONS

The presence of a diverse microbiome, both beneficial and pathogenic, was detected on grapes in Javgur of the Cimișlia district. Thus, it was concluded that the grapes from the mentioned region have yeasts from the *Saccharomyces* genus that allow spontaneous fermentation to take place on indigenous yeasts. This fact would ensure a decrease of expenses, but beside fermentation yeasts, other types of yeasts (*Brettanomyces*) and even bacteria (*Acetobacter*) that could danger the fermentation process were detected in the analysed samples.

An efficient use of the grape microbiome would be the selection of specific microorganisms and their subsequent multiplication to be used for fermentation (starter cultures), thus, this could possibly limit problems regarding the infection of the wine with other types of microorganisms which are contained on the grapes, and more the authenticity of the wine from the specific geographical area is preserved.

However, if spontaneous fermentation on native yeasts is an option, it is necessary to carry out systematic monitoring of the fermentation process, in order not to admit the triggering of unwanted processes, because the use of indigenous yeasts does not allow obtaining good reproducibility of the must microbiome and, therefore, of consistent wine quality, leaving much room to competition between strains and random contamination.

Thus, the spontaneous fermentation of the grape must could be followed by a number of disadvantages, such as: obtaining wines with an incomplete fermentation; the danger of wine contamination with pathogenic flora; low alcohol content; high content of volatile acids; slower wine clarification comparing to wines produced by inoculation of selected yeast cultures.

Therefore, it is important to mention that the inoculation of a starter culture could reduce the latency time before starting the must fermentation, as well as other advantages listed above, especially compared to spontaneous fermentation.

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