RESEARCHES CONCERNING THE EVOLUTION OF WINE MICROBIOTA DURING THE SPONTANEOUS FERMENTATION OF RED GRAPES JUICES

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Abstract
In order to keep its place on the wine international market, Romania should produce typical wines starting from local grape varieties and conducting the alcoholic fermentation by starter cultures obtained from local isolated wine yeast. The present study was initiated due to the fact that there are few informations regarding the evolution of wine microrganisms in plantations and in the fermentations process for quantitative point of view.
The population dynamics of microbiota in Valea Calugareasca vineyard was analysed during the alcoholic fermentation. No yeast starter cultures had been used in order to investigate the dynamics of grape-related indigenous microorganisms population. Classical works and methods for alcoholic fermentation monitoring have been employed at this level.
At the beginning of fermentation the total number of yeasts found is doubling, while the number of bacteria is stabilized at a value of 10^3 CFU/ml. During the alcoholic fermentation the yeasts become predominant (10^7–10^8 cfu/ml) and continue the fermentation until its completion. Significant differences regarding the evolution of yeast microbiota for quantitative point of view between varieties have been recorded.

Keywords: grape juice, spontaneous fermentation, fermentation speed

1. INTRODUCTION

The yeasts and the bacteria are microorganisms with a considerable influence about quality and value of wine [1], [2]. The yeasts guide the alcoholic fermentation and contribute to achievement of the main composition of wine and typicality of the wine savour and fragrance. The proportions of this aspect belong to the yeast species and strains implicated in alcoholic fermentation.
The yeasts are microorganisms which are frequently used in the classical or modern biotechnological processes [3]. In winemaking process the yeasts could lead to the depreciation of wine quality and consequently the decrease of wine value, after the alcoholic fermentation.
The main bacteria associated with winemaking are lactic acid bacteria and acetic acid bacteria. The different categories of bacteria contribute to improve the quality of wines or to unwanted modification of it [6].
The lactic acid bacteria, especially the strains of *Oenococcus oeni*, are responsible for malolactic fermentation, which lead to reduce acid in wine, improve the flavour and increase the microbiological stability. Malolactic fermentation, after the alcoholic one, is the key of secondary fermentation for many wines. Besides *O. oeni*, other lactic acid bacteria can growth in wine but, in generally, these could induce the damage of wine. The acetic acid bacteria are microorganisms which lead to the wine depreciation; they mainly contribute to increase the volatile acidity (acetic acid), to form aldehydes, esters and to destroy the flavour. High levels of lactic acid bacteria and acetic acid bacteria on grapes and in must could determine a slow alcoholic fermentation and even stop it. Other bacteria, with a small appearance frequency, could determine problems during the fermentation of the must or diseases of wines. The development conditions determine the useful or harmful nature of wine microorganisms and this is the main problem for the oenologist [7].
The yeasts and the bacteria implicated in winemaking process proceed from more sources, like:

(1) microbiota from grapes surface;

(2) microbiota associated with the surface of equipment used in winemaking (initially proceed from grapes surface) and

(3) like starter or inoculated yeast cultures used in alcoholic fermentation, or *O. oeni* used to induce malolactic fermentation.

A modern oenology recognises the impact of diversity and complexity of microbiota and the necessity to understand and to manage the implication of microorganisms in winemaking process.

The microorganisms associated to winemaking process are significant from more points of view. First, the grapes are the main source of yeasts and bacteria which contribute to wine fermentation or depreciation process. The second, these could affect the grapes and must quality through depreciation reactions before collect.

Although the filamentous funguses are considered the most important group in this circumstance, the yeasts and the bacteria also contribute to certain grapes deterioration types. In addition, a few species of yeasts and bacteria on the grapes surface have an important antifungal activity and could be useful as natural modulator for funguses which induce alteration. There is an increasing interest for using this species as biocontrol agents, to decrease utilization of chemical fungicides in vineyards and to reduce the associated environmental risks. The grapes are a natural resource of biodiversity and represent a reservoir of strains which can be used as novel organisms to achieve alcoholic and malolactic fermentations.

In spite of its importance, vine and wine microbiology is so little understood in wine science field [2]. Although many researches reported the isolation and identification of yeasts and bacteria (as far as possible), there are a few studies regarding the quantitative evolution of these microorganisms in vineyard and in winemaking process.

Such as information are essential for manage the grapes quality and also for understanding the origin of significant microorganisms in winemaking process.

2. MATERIAL AND METHODS

The grape samples used for spontaneous fermentation was collected from Dealu Mare vineyard, Valea Călugărească vine centre, important vine area for red wines production.

The biological materials were red wine grape variety recommended in Valea Călugărească vine centre: Cabernet Sauvignon, Burgund mare, Merlot, Fetească neagră and Pinot noir.

The grapes were collected with pedicel, using sterile instruments and vessels.

For establishing a grape and followed must microbiota dynamic, the samples were prepared in this way:

- first step: the grapes were aseptic collected, put in the flask and covered with sterile distillate water; the samples were agitated time to time; after 24 hours, the suspension was inoculated on the specific media for quantitative determination of the grapes surface microbiota. The YEPD (Yeast Extract Peptone Dextrose) medium for yeasts, the Lafon – Lafourcade medium for lactic acid bacteria and the Carr medium for acetic acid bacteria were used. The composition of these culture media is presents in Table 1.

Table 1. The culture media chemical composition

Substance	Amount (1000 ml)		
	YEPD	Lafon - Lafourcade	Carr
Yeast extract	10 g	5 g	30 g
Meat extract		10 g	
Peptone	20 g	15 g	
Sodium acetate		5 g	
Magnesium sulphate		0.2 g	
Glucose	20 g	20 g	
Chloramphenicol	100 mg		
Actidione		0.05 g	50 mg
Bromocresol green 2.2%			1 ml
Agar	20 g	20 g	20 g
Ethylic alcohol			300µl add to 15 ml medium before utilisation

- second step: the grapes were squash with a sterile clip and the grape juice has

fermented at the 20ºC; inoculation to three the first inoculation at the beginning of fermentation (24 hours); the second inoculation at the middle of fermentation period (72 hours) and the third at the end of fermentation (10 days). After the must decimal dilutions were inoculated in three different moments were realised from must: repetitions, the plates were incubated at 28ºC for 24-48 hours and then the colonies were counted with Funke - Gerber colony counter and the colony forming units (CFU) per ml was established (Figure 1).

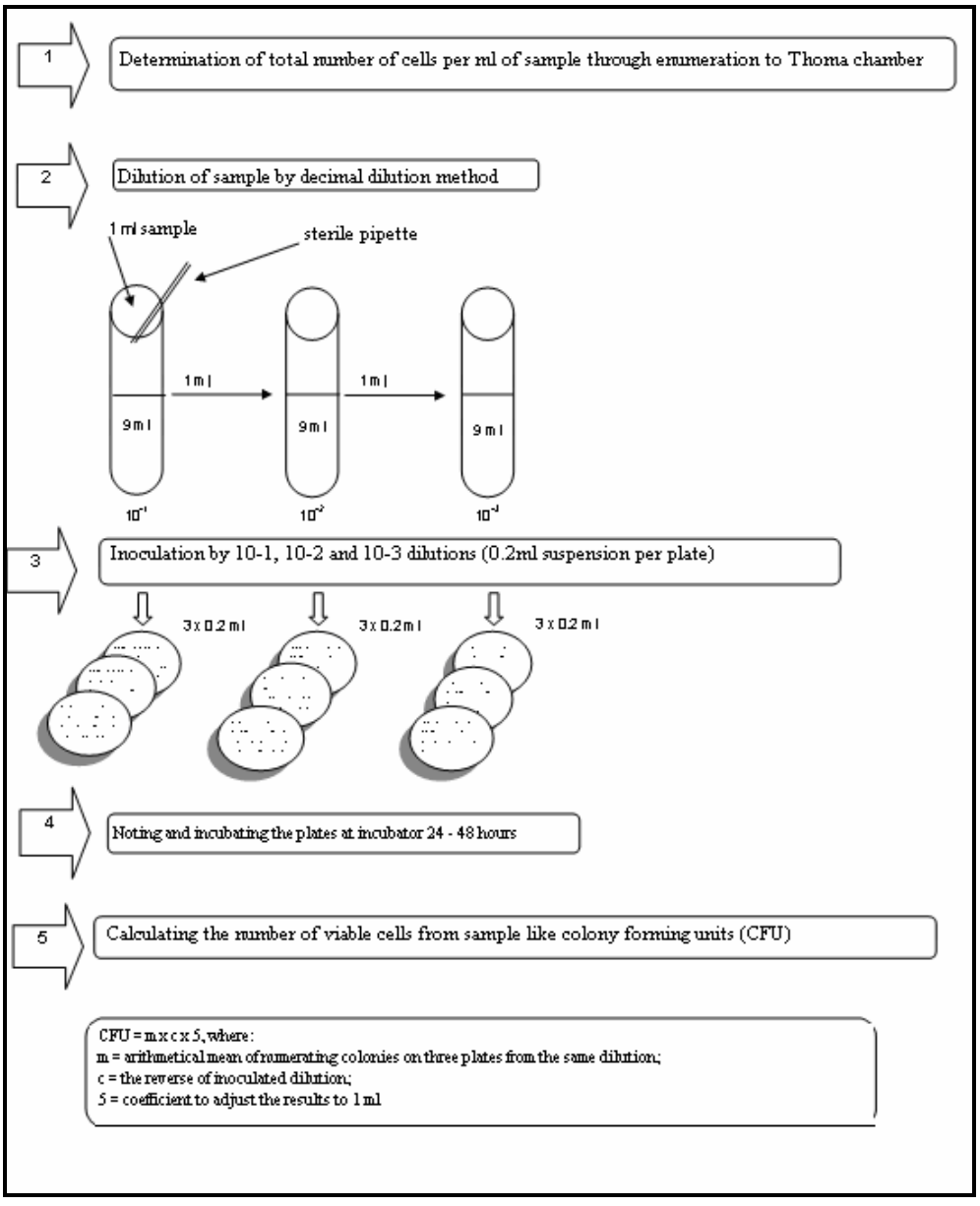

Figure 1. The decimal dilution method for establishing CFU

For the evaluation of sugar content in must, optical methods (refractometrical, polarimetrical, colorimetric methods) or densimeter and enzymatic methods are used. Although the densimetrical methods are more

precisely, the refractometrical method is the only official method accepted by OIV.
For the study of evolution for grape maturation process, a hand refractometer (Zeiss) was used. It is a monocular device, which measured the percent of dry matter soluble in must (between 0 and 30 %). It was calibrated at 20°C.
After the grape juices obtaining, a few drops was take from clear must and was put on the refractometer prism. The 0 gradation was verified with double distilled water. The obtained dates were corrected function on the temperature (20°C). The sugar percent from must was established by formula:

$$\text{Total sugar \%} = N*4.25/4 - 2.5 \text{ (g/100ml)}$$

where N represents the dry substance from must, in %.
The value of the sugar in must is usual about 2-5 g/l. During the alcoholic fermentation, the sugar is metabolized by yeasts. The hydrolyze process is made by enzymes and catalyzed by tartaric acid. In must, this enzyme is *the invertaza*, a levurian enzyme, with optimal activity in acid media (as must, pH = 3-4).
In this work the sugar content in must and the fermentation speed were calculated.

$$v = (C_{10} - C_3)/t$$

Where: t is studied time of fermentation process (7 days = 168 hours), C_{10} and C_3 are the concentration of the sugar in must after 10 days (respectively 3 days) from fermentation initializing.
For a better characterization of fermentation, calculated fermentation speed could be associated with CO_2 and ethylic alcohol production, like final products [4].

3. RESULTS AND DISCUSSION

The microbiota quantitative determinations for the grapes surface and the spontaneous fermentation are presented in next figures. In figure 2 is illustrated the dynamic of yeast multiplication begin to grapes microbiota till the end of fermentation.
The Cabernet - Sauvignon must developed a very intense multiplication of yeasts during a spontaneous fermentation, especially the middle and the end period, by comparison to the other musts used in experiment, with lower values of yeast microbiota.

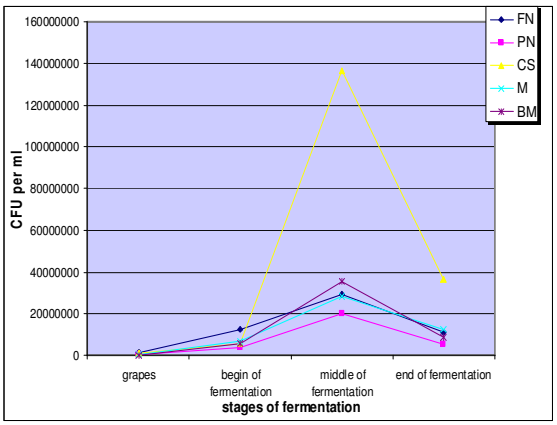

Figure 2. Dynamic of yeast multiplication during spontaneous fermentation

In figure 3 and figure 4 is showed the evolution of bacterial microbiota for studied musts, starting from grapes microbiota to the end of spontaneous fermentation. The lactic acid bacteria presented a similar dynamic of multiplication for all five musts, according to preliminary values for initializing fermentation.

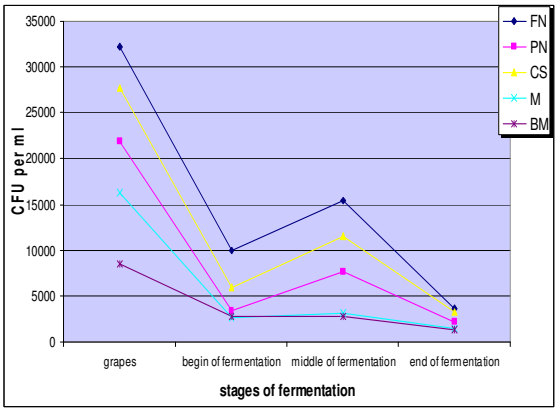

Figure 3. Dynamic of lactic acid bacteria multiplication during spontaneous fermentation

The quantity of lactic acid bacteria was between $8.5x10^3$ CFU/ml for Burgund mare and $3.2x10^4$ CFU/ml for Feteasca neagră on the grapes, but the values registered for early period and for the end of alcoholic fermentation were low, below $1.0x10^4$ CFU/ml. The higher values were registered for Feteasca neagră must, the lower values for Burgund mare (Figure 3). The quantity of lactic acid bacteria was between $8.5x10^3$ CFU/ml for Burgund mare and $3.2x10^4$ CFU/ml for Feteasca neagră on the grapes, but the values registered for early period and for the end of alcoholic fermentation were low, below $1.0x10^4$ CFU/ml. The higher values were registered for Feteasca neagră must, the lower values for Burgund mare (Figure 3).Regarding the evolution of acetic acid bacteria during the studied fermentations, all the used musts presented a gradual reduction of acetic microbiota. The most pronounced reduction of bacterial amount was observed for Merlot must, from $1.4x10^4$ CFU/ml on the grapes to 0 for the end of fermentation process (Figure 4).

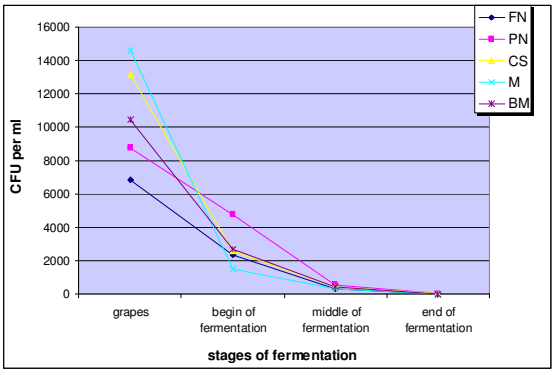

Figure 4. Dynamic of acetic acid bacteria multiplication during spontaneous fermentation

To the aim of establish the sugar content and also for the estimate the fermentation speed, the measure of studies musts refractive index was used.

The dates obtained for the sample, after 3 days from the fermentation initializing, are related in table 2:

The refractive index and sugar content, after 10 days from the fermentation initializing, were presented in table 3.

The fermentation speed between the third and the tenth day of fermentation was illustrated in table 4.

Table 2 The sugar content after three days

The sample	dry substance from grape juice (N) %	The sugar content g/100ml
Feteasca neagră	24.1	23.1062
Pinot noir	24.3	23.3187
Cabernet Sauvignon	23.9	22.8937
Merlot	26.9	26.0812
Burgund mare	24.0	23.0000

Table 3 The sugar content after ten days

The sample	The refractive index (N) %	The sugar content g/100ml
Feteasca neagră	13.9	12.2687
Pinot noir	16	14.5000
Cabernet Sauvignon	18	16.6250
Merlot	15.5	13.9687
Burgund mare	7.3	5.2562

Table 4 The fermentation speed between the third and the tenth day

The sample	The fermentation speed
Feteasca neagră	0.0645
Pinot noir	0.0525
Cabernet Sauvignon	0.0373
Merlot	0.0720
Burgund mare	0.1056

The dates related in table 4 illustrated that the highest fermentation speed between the third days to the end of fermentation was registered for Burgund mare must, an amount by 17.7438g/100ml saccharum was metabolized. The smallest value was registered for Cabernet Sauvignon must; in that case, the efficiency of sugar metabolism was low, only by 6.2687g/100 ml, although the yeast

microbiota was abundant at the middle of fermentation.

4. CONCLUSIONS

Analyze of dates obtained in these experiments emphasized the presence of certain microbiota on the grapes and in the must, capable to determine the spontaneous fermentation in proper conditions, without sugar supplement.

The evolution of yeasts, lactic acid bacteria and acetic acid bacteria during the spontaneous fermentation process suggests they could be used in strain selection for controlled fermentations.

The number of yeast cells on the grape surface at complete maturation wasn't significantly different for all five varieties. The values for total number of lactic acid bacteria and acetic acid bacteria were between 10^3 and 10^4 CFU/ml.

We found that the number of yeast was doubling at the beginning of fermentation, while the number of bacteria stabilized by 10^3 CFU/ml.

During the alcoholic fermentation, the number of yeast cells increases to 10^7-10^8 CFU/ml, but the number of lactic acid bacterial cells decreases and the acetic acid bacteria disappear almost entirely.

The must fermentation speed was different for those five samples, although the process began from close values of sugar.

5. REFERENCES

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