# Data concerning the yeasts microbiota from Cotnari vineyard

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## Abstract

*Nowadays to know the spontaneous yeasts microbiota represents a very important desideratum not only for strictly research activity so as practical operation with direct implication in wine industry.*

*Based on this aspect it was necessary to begin a series of studies concerning the composition of the yeasts spontaneous microbiota.*

*This study developed between 2006 – 2007 in the Cotnari vineyard followed yeasts isolation from grapes and its persistence on musts. Also, was made a study regarding yeasts incidence and its dynamics on grapes and musts in the early or late stage of fermentation.*

*In accordance with results the conclusions was that* ***Ascomycota*** *species has prevailed compared with* ***Deuteromycota****; the prevalent* ***Ascomycota*** *species was* ***Saccharomyces ellipsoideus;*** *the prevalent non -* ***Saccharomyces*** *species was* ***Hansenula anomala****.*

*It also was registered many differences between species isolated from grapes and that one was isolated from musts regarding occurrence of its or number. The explanation consist in poor yeasts number on grapes and also possible contamination from vinery equipments.* ***Candida mycoderma*** *was noted as ubiquitous species based on its presence on grapes after that in musts and above all in wine.*

Key words: Cotnari vineyard**,** microbiota yeasts, grapes, musts, identification

## Introduction

Many ecological studies have been made in most vineyards from Romania, so that nowadays the yeasts prevalence and frequency on grapes is well known [1], [15], [16], [19], [25], [27], [28].

It is also established that the soil is the common habitat for yeasts and could contain 25 x $10^3$ yeasts cells per gram, prevailed in the first 30 - 40 cm depth.

From soil, by wind or other physical or biological vectors the yeasts coming up to leaves, clusters or grapes. By the other hand, the insects have one significant role especially bee, wasps or just *Drosophila melanogaster* that can realize the transfer of yeasts cells between grapes and also can assure the life cycle of yeasts. The rate of yeasts species occurred on grapes may vary in connection to climatic conditions.

As far as yeasts occurrence is concernned, T. Castelli [11] has studied grapes from different regions of Italy and observed massive prevalence of *Kloeckera apiculata*.

I. Benda [5] as results of his research noticed that *Candida pulcherrima* prevail on grapes in rainy years.

Investigation by electronic microscope revealed yeasts occurrence, especially on upper part of the grapes and also on the grape skin which surrounding stomata [4]. Also, have been well established that no fermentation process occurs inside of grapes but only if the skin is intact. All of those arguments clearly demonstrate the presence of yeasts only on the surface of the grapes and no inside it. In the musts obtained from healthy grapes the number of yeasts cells could be $10^3$ - $10^5/cm^3$. By the other hand, is clearly demonstrated that from grapes with affected skin and especially if there is loss in juice, will result one significant increase in yeasts cells number [6], [7].

At present, there are many arguments to this extent. Thus, J. Sturm has compared the yeasts obtained by direct isolation in vineyard, from grapes and yeasts isolated from musts. His conclusion was that there are significant differences in yeasts number namely in musts where yeasts number was superior [26]

With grapes, there appear many yeasts genera, but *Kloeckera* and also *Saccharomyces* are dominant species.

Domercq, mentioned by P. Ribéreau-Gayon [23], has analyzed up to 2000 yeasts strains isolated from harvest between 1951 and 1983. He has concluded that 64% was sporogenous, *Saccharomyces cerevisiae* var. *ellipsoideus* was dominated, followed by *Saccharomyces bayanus*, *Saccharomyces rosei*. Among non-sporogenous species *Kloeckera apiculata* (78%) and *Torulopsis bacillaris* (19 %) was dominated. On red grapes most frequent was *Saccharomyces chevalieri* with fermenting properties just like *Saccharomyces cerevisiae* var. *ellipsoideus*.

On moulded grapes, *Torulopsis bacillaris* was dominant, because of its tolerance to some antibiotic substances produced by moulds.

Although grapes yeasts microbiota is qualitative and quantitative different regarding oenological role, the yeasts being classified in many groups [12].

During fermentation, the microorganisms initially occurred in grapes must, depending on its physiology and musts composition, are organized in some succession according to ecological relation developed between different microbial groups [9], [14], [20]. Musts fermentation is initiated by anascosporogenous yeasts such as *Kloeckera apiculata* and *Torulopsis bacillaris* for white grapes musts, while *Kloeckera apiculata* dominate in early stage of red grapes musts fermentation.

The musts fermentation could be initiated by other yeasts species with low alcohol tolerance such as *Hanseniaspora* and *Candida*.

As a rule, spontaneous musts fermentation is initiated by “contamination” of musts with yeasts that exists on vinery equipment. Some genera like *Kloeckera, Hanseniaspora, Candida*, *Pichia*, and sometimes *Hansenula* could have notable growing in the early stage of fermenting process. After that, this “initial yeasts” decreases in numbers and alcohol tolerant species such as *Saccharomyces cerevisiae* gain ground finishing fermentation [18].

In spontaneous fermentation there is some antagonic relation between microorganisms, especially in unfavorable condition (poor aeration, high temperature etc) because of inhibitory substance released by some yeasts.

For example, a part of yeasts strains has killer activity expressed by some polypeptide toxins that kill sensitive strains of the same genus or less frequently strains of different genera [8], [22], [24]. Among wine yeasts usually founded in grapes must only 2 - 3% can produce killer toxins. Otherwise, this aspect has been observed for some genus like *Saccharomyces*, *Torulopsis*, *Candida,* and *Pichia* [13]. The experiments have clearly demonstrate that in fermentation with a mix of killer and non-killer yeasts strains (1/20 ratio), the process will be always finished by killer strains.

Some researches in this domain established that killer toxins are unstable at temperature, for example at $45^0$ C the toxins are destroyed in 15 minutes. On the other hand, if toxins pass into wine, then it can keep its characteristic up to 6 months at $4^0$ C.

Recently has been described a series of antagonistic interaction between yeasts and other species of microorganisms. For example *Rhodotorula* sp. a prevailed genus in majority of habitats, can produce substances with inhibitory effect for *Aspergillus niger*.

Also, grape musts obtained from moulded grapes is possible to contain antibiotics like botryticina, with inhibitory effect for yeasts. After some investigations it has established that botryticina become inactive after heating ($120^0$ C) or in contact with sulphur dioxide.

Finally, the conclusion is that fermenting grape musts are really microbial ecosystems with complex interaction between different components, species or genus actually, which can growth or die function of its adaptability against environmental condition.

V. D. Cotea & all. in the important monograph *Podgoria Cotnari* has described with accuracy and professionalism, Cotnari vineyard and its surroundings [17]. This interesting scientific book offers a very useful and completely informations by geographical or ampelographical angle and in the same manner a realistic introspection in historical or social aspects.

Cotnari vineyard and surroundings is situated in a tempered zone which make transition between pronounced continental climate (Eastern European) characteristic for Moldavian bent and gentle continental climate (Central European).

Solar radiation like basic energy source has 118.87 kcal/cm$^2$ for Cotnari, the most important radiant energy value for entire northern area of Moldavian Plateau.

Generally, on the slopes with angle 5 – 15 % and eastern exposition, solar radiation has value up to 120 - 140 kcal/cm$^2$. In the same time, for southern slope radiation is up to 130 - 150 kcal/cm$^2$ so there are different functions of exposition and slope angle. Anyway, the most disadvantageous connected with solar radiation was slope with Northern and North Western exposition where radiation value could be down to 100 kcal/cm$^2$, as it turned out.

Finally, caloric balance in course of one year has positive value in March - October (gain of radiation is major compared with loss and a climax in June - July) and negative value in November-February when loss of radiation is up to gain in it (minimum in December – January).

Duration of solar brightness – has a medium annual value of 2100 hours. Up to 2/3 from this value is dispensed between April and September, when total insolation summarizes 1448 hours (69%) compared with 1/3 in cold season (638 hours).

Temperature is an effect of solar radiation conversion in thermal degree. For Cotnari vineyard it depends of altitude and atmospheric dynamics; for this vineyard area average value for temperature is rather high namely $9^0$ C.

Medium thermal amplitude derive as a results of average value for extremely cold months ($-3,5^0$ C in January) and warm months ($+20,2^0$ C in July). By this point of view thermal amplitude is moderately for Cotnari compared with surroundings (Iasi, for example, has registered 24,7 $^0$C thermal amplitude).

Extreme temperature registered for Cotnari was – $24^0$ C (1972) and + $36^0$ C (1988).

Medium number of frost days is down to surroundings 105,4 days per year. Media of registered summer days (≥ $25^0$ C) is 55 and tropical days (≥ $30^0$ C) are 9 days. These both value relieve a moderately thermal amplitude especially because altitude such as to avoid eventually thermal excess.

Soil temperature, a very important parameter, registered in media $10.3^0$ C for the Cotnari vineyard.

Precipitation media per year is 520 mm. with maximum value in June (78.8 mm) and minimum in January (23.1 mm).

## Material and Methods

This study has taken place in Cotnari vineyard between 2006 - 2007, following yeasts isolation and identification from spontaneous microbiota, as the first contribution of the complex study regarding a wider biodiversity research, some of the isolated strains are going to be further tested for their oenological and biotechnological potential. We also have taken notice concerning yeasts incidence on grapes or fermenting musts.

The grapes were harvested from the Cotnari vineyard has belonged to representative grapes sorts for this area and it was: Frâncuşă „Terente", Grasă de Cotnari „Plăieşu" and Tămâioasă Românească „Paraclis".

**Table 1.** Geographical characteristics of harvest stationary

| Nr. crt | Harvested grape sample | Slope exposure | Altitude (meters) |
|---|---|---|---|
| 1. | Frâncuşă „Terente" | Eastern | 238 |
| 2. | Grasă de Cotnari „Plăieşu" | Eastern | 246 |
| 3. | Tămâioasă Românească „Paraclis" | South- eastern | 252 |

The samples for this study was harvested from representatively stationary for the Cotnari vineyard namely slope with Southern or South - Eastern exposure (Table 1).

The musts resulted from the same type of grapes was processed in industrial and laboratory condition too.

Yeasts isolation from grapes: clusters of grapes were harvested from different location. All of grapes belonged for characteristic variety of grapes for Cotnari: Frâncuşă „Terente", Grasă de Cotnari „Plăieşu" and Tămâioasă Românească „Paraclis".

The grapes was cut out from clusters and mixed in a blender for 2 minutes approximately, to obtain juice. From this juice was made dilution between $10^{-1}$.....$10^{-6}$, each of its becoming inoculums on Petri plates with selective media, for obtain insulated culture. This selective media was Sabouraud with cloramphenicol (Biomedics Laboratory, Madrid Spain). The plates Petri with inoculum were maintains al $27^0$ C for 3 days.

Yeasts isolation from musts: samples were taken from different musts raised from healthy grapes. The samples were let to ferment spontaneously. The yeasts isolation was made in the beginning of fermentation when, as it is well known, there are abundant yeasts microbiota.

The second isolation was made in the end of fermentation when there is a high percent of alcohol. It was used the same selective medium Sabouraud with cloramphenicol in Petri plates. After those plates was placed at $27^0$ C for 3 days.

Another relatively easy method used in this study for the isolation of yeasts, involves direct collection of grapes in the sterile bags. This method is first advantageous because it is easy to realize and offers many advantages, considering yeasts incidence on grapes for different stationary. Disadvantages for this method could be contamination risk, which exists especially for direct collecting in stationary. This disadvantage could be in part avoided by using some instruments for cutting grapes (scissors or another) and preferably gloves. After a sufficient quantity of grapes is collected (1.5 - 2 kg usually) the samples should be closed and carry in laboratory. After that, grapes collected must be crushed such as to obtain juice (crushing is done in the same bags) and incubated at 25 - $27^0$ C for yeasts growing (because as it well known a small number of yeasts naturally exists on grapes) [3].

Yeasts strain purification: each of insulated colonies results after preceding steps, was successive reinoculated on adequate media (Yeasts Peptone Glucose Agar) until uniformity has been reached. Accordingly uniform colony and relative pure strains has been obtained. The species obtained as a result of purification was identified by classical method [21] based on its morphological and physiological characteristics and in added with that by modern method (API 20 gallery, micro test -Biomerieux Laboratory Lyon France).

## Results and discussion

As results of this study, a number of 250 yeasts strains from grapes as well as musts have been isolated and partially retained. Regarding musts yeast strains, isolation was performed in early and late stage of fermenting process. The identified yeasts species has belonged to *Ascomycota* (dominant) and *Deuteromycota*.

After isolation and identification it has been observed a prevalence of *Deuteromycota* especially on grapes or in early stage of fermentation. Once a sulphur dioxide treatment has been applied on grapes or in musts, *Deuteromycota* yeasts species dramatically decrease in number (Table 2). In added of that, has been noted persistence of *Candida mycoderma* in the end of fermentation.

According to the results presented, the *Ascomycota* yeasts species has a notable prevalence, when compared to the *Deuteromycota* yeasts (Table 3). Among the *Ascomycota* yeasts, the *Saccharomyces* genus is most prevailed. The predominant species was *Saccharomyces ellipsoideus*. Adjacently, we identified *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces oviformis*.

At the beginning of the fermentation, the ratio between species isolated from grapes and that which was isolated from musts was quite constant. In this case, some species, which has not been isolated from grapes, could be present on musts. One possible explanation for that resides in a poor yeasts occurrence on grapes or because of possible contamination from vinery equipments.

The most prevalent *non-Saccharomyces* species was *Hansenula anomala* one of the ubiquitous species in the wine industry. Besides of this species some others like *Pichia membranifaciens* (= *Saccharomyces membranifaciens*, *Debaryomyces membranifaciens*) and also *Kluyveromyces sp.* could be present. Significant decrease in number for *Deuteromycota* yeasts species in early stage of fermentation could be due to sulphur dioxide treatments applied on grapes. Beside of that, the alcohol accumulation must be taken in considered, knowing that the many yeasts species are sensitive to alcohol content. The difference concerning some species occurrence in the early stage of fermentation compared with late stage could be due to progressive alcohol accumulation on the fermenting process [10].

The occurrence of *Brettanomyces* on grapes was not related for a while, but lately many researchers reported its presence on grapes [3]. Anyway, *Brettanomyces* wine occurrence is traditionally referable to contamination by vinery equipments. On the other hand, *Brettanomyces* most occurred in red vinification and was a long time considered “typical infection yeast”, but in the future will be possible to revising that.

**Table 2.** The species and strains yeasts microbiota from ***Deuteromycota***

| Nr. crt. | Species | Strains number isolated from grapes variety | | | Strains number isolated in early stage of fermentation | | | Strains number isolated in late stage of fermentation | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Francuşă “Terente” | Grasă de Cotnari “Plăieşu” | Tamâioasă Românească “Paraclis” | Francuşă “Terente” | Grasă de Cotnari “Plăieşu” | Tamâioasă Românească “Paraclis” | Francuşă “Terente” | Grasă de Cotnari “Plăieşu” | Tamâioasă Românească “Paraclis” | |
| 1. | ***Candida mycoderma*** | 3 | 2 | 2 | 1 | 1 | - | 1 | 1 | - | 11 |
| 2. | ***Hansenula uvarum*** | 4 | 7 | 4 | 1 | 2 | 1 | - | - | - | 19 |
| 3. | ***Torulopsis stellata*** | 3 | 3 | 3 | 3 | 3 | - | - | - | - | 8 |

**Table 3.** The species and strains yeasts microbiota from ***Ascomycota***

| Nr. crt. | Species | Strains number isolated from grapes | | | Strains number isolated in early stage of fermentation | | | Strains number isolated in late stage of fermentation | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Frâncuşă „Terente” | Grasă de Cotnari „Plăieşu” | Tămâioasă Românească „Paraclis | Frâncuşă „Terente” | Grasă de Cotnari „Plăieşu” | Tămâioasă Românească „Paraclis” | Frâncuşă „Terente” | Grasă de Cotnari „Plăieşu” | Tămâioasă Românească „Paraclis” | |
| 1. | ***Saccharomyces cerevisiae*** | 1 | 2 | - | 1 | 2 | - | 1 | 1 | - | 8 |
| 2. | ***Saccharomyces ellipsoideus*** | 2 | 3 | 2 | 1 | 1 | 1 | 1 | - | 1 | 12 |
| 3. | ***Saccharomyces oviformis*** | 1 | - | 1 | 1 | 1 | 1 | 2 | 1 | 3 | 11 |
| 4. | ***Saccharomyces bayanus*** | - | 1 | 1 | 1 | 2 | - | 1 | 1 | 1 | 8 |
| 5. | ***Saccharomyces chevalieri*** | - | 1 | - | - | - | 1 | - | - | 1 | 3 |
| 6. | ***Saccharomyces heterogenicus*** | 1 | - | 1 | 1 | - | - | - | - | - | 3 |
| 7. | ***Saccharomyces kluyveri*** | 1 | - | - | - | 1 | - | - | - | - | 2 |
| 8. | ***Saccharomyces uvarum*** | - | 1 | - | 1 | - | - | 1 | - | - | 3 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **9.** | ***Saccharomyces italicus*** | - | - | - | - | - | 1 | - | - | - | 1 |
| **10.** | ***Saccharomyces baillii*** | 1 | - | - | - | 1 | - | - | - | - | 2 |
| **11.** | ***Saccharomyces florentinus*** | - | - | - | - | - | 1 | - | - | 1 | 2 |
| **12.** | ***Saccharomyces rouxii*** | - | - | - | - | 1 | - | - | - | - | 1 |
| **13.** | ***Kluyveromyces* sp.** | 1 | - | 1 | - | - | - | - | - | - | 2 |
| **14.** | ***Pichia membranefaciens*** | 1 | 1 | 1 | - | - | 1 | - | - | - | 4 |
| **15.** | ***Hansenula anomala*** | 2 | 1 | 1 | 1 | - | 1 | - | - | - | 6 |
| **16.** | ***Brettanomyces* sp.** | - | - | - | - | - | - | - | 2 | 1 | 3 |

Also, must be mentioned the apart case, well-know in the Cotnari vineyard, too, constituted by the wine obtained from botrytized grapes, namely obtained from grapes infected with *Botrytis cinerea* [2].

The phenomenon has as consequence the obtaining of high sugar content musts, usually up to 250g/l. In fermentation process in the musts resulted from botrytized grapes can be registered significant difference in yeasts microbiota compared with common musts (was reported one notable increase for *Candida sp.* and *Kloeckera sp.*)

## Conclusions

In the period 2006 – 2007 in the Cotnari vineyard, 250 yeasts strains from grapes and musts belonging *Ascomycota* (dominant) and *Deuteromycota* have been isolated. Among *Ascomycota* yeasts, *Saccharomyces* genus is most prevailed. The predominant species was *Saccharomyces ellipsoideus*. Adjacently, was identified *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces oviformis*.

At the beginning of the fermentation, the ratio between species isolated from grapes and musts was quite constant, but in this case, some species, which has not been isolated from grapes, could be present on musts.

The most prevalent *non - Saccharomyces* species was *Hansenula anomala* one of the ubiquitous species in wine industry. Besides of this species some others like *Pichia membranifaciens* (= *Saccharomyces membranifaciens*, *Debariomyces membranifaciens*) and also *Kluyveromyces sp.* could be present.

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