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The assessment of initial number of yeasts and lactic acid bacteria in some Albanian grape varieties compared with other varieties used for wine production

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Abstract

In this study we have done wine with Albanian grape varieties like Kallmet, Black Shesh, White Shesh, Pulz, and other grape varieties like Merlot and Kabernet. There are many microorganisms in grape that are used to do wine. Some of them are wild yeast, lactic acid bacteria, and acetic acid bacteria. Yeasts that are in grape are indigenous yeasts and spontaneous fermentation is done by them.

In Albania, some of wine producers produce wine with spontaneous fermentation and some other produce wine with inoculated yeasts that are *Saccharomyces cerevisiae, Saccharomyces bayanus* or a mix between two yeasts, *Saccharomyces cerevisiae* and *Sacharomyces bayanus*. The aim of this study is to evaluate the initial number of yeasts and lactic acid bacteria in these grape varieties, to compare the quantity of these microorganisms between these grape varieties, to evaluate the difference between yeasts and lactic acid bacteria and their performance during the two fermentations, (spontaneous and inoculated fermentations), and the effect of yeasts to lactic acid bacteria.

Key words: wine, yeast, lactic acid bacteria, initial number, spontaneous and inoculated fermentations.

Introduction

There are many microorganisms in grape that are used to do wine. Some of them are wild yeast, lactic acid bacteria, and acetic acid bacteria. Yeasts that are in grape are indigenous yeasts and spontaneous fermentation is done by them.

Wine fermentation is, as in many other food fermentations, characterized by complex chemical and microbial interactions. Lactic acid bacteria and yeast are the first to develop after crushing of grapes. Bacterial numbers increase to 10^3 or 10^4 cfu ml"^{1}, but decline to almost undetected levels during alcoholic fermentation [14]. *Oenococcus oeni, Leuconostoc mesenteroides* subsp. *mesenteroides, Lactobacillus plantarum, Lactobacillus casei* and *Pediococcus damnosus* (previously *Pediococcus cerevisiae)* are major species present [2]. At the end of alcoholic fermentation, growth commences and cell numbers increase to approx. 10^7 cells ml^{-1}. *O. oeni* usually predominates in wines of low pH (<3.5), while *P. damnosus* grows in wines with higher pH resulting in spoilage of wine [21]. Yeast numbers at time of harvest range between 10^4 and 10^6 cfu g^{-l}. The most predominant genera are *Rhodotorula, Cryptococcus, Candida, Hanseniaspora Metschnikowia* and the yeast-like fungus *Aureobasidium pullulans* [5]. As alcoholic fermentation commences, growth of species less resistant to ethanol, e.g. *Hanseniaspora, Candida, Pichia, Kluyveromyces, Metschnikowia* and *Issatchenkia,* is suppressed and *Saccharomyces cerevisiae* proliferates [2, 21, 24].

The secret to good winemaking is to manage the process in such a way that interactions between yeast, lactic acid bacteria, and yeast and lactic acid bacteria are controlled at all times. This may be difficult, as seen with stuck or sluggish malolactic fermentations and the sudden production of off-flavours. Many underlying factors may be responsible for this, including viticultural practices, such as

spraying of the grapes with fungicides, high levels of flavenoides and phenolic compounds in grapes, or flavenoides and phenolic compounds released from oak barrels during maturation [3, 11, 21].

At the onset of fermentation, yeast and bacteria are in fierce competition for nutrients. Both groups have developed unique survival strategies to compete against each other and cope with conditions in a rather extreme environment. *S. cerevisiae* has a very active metabolism, tolerates oxygen and relatively high levels of $S0_2$. During alcoholic fermentation, some strains produce $S0_2$, short chain fatty acids, peptides, proteins or glycoproteins, such as killer toxins, and lytic enzymes that are used to inhibit the growth of malolactic bacteria [1, 12, 25].

Lactic acid bacteria, on the other hand, are slow growing, fastidious and require adequate levels of carbohydrates, amino acids and vitamins to survive. Like yeast, they prefer acidic environments. It is thus understandable that they have, over the years, developed an arsenal of antimicrobial compounds to compete with faster growing organisms, including yeast. Many lactic acid bacteria, including malolactic species, produce bacteriocins against other bacteria. A few lactic acid bacteria have also developed the ability to produce antifungal compounds

It is clear that organisms forced to co-survive in the same habitat have developed unique forms of "communication". We are only beginning to understand the regulation of enzymatic reactions and competition between microbial cells through quorum sensing. Most of these studies have been done on microorganisms in niches other than wine [4, 5, 14, 9, 16, 21].

1.1Yeast-Bacteria Interactions

Malolactic fermentation usually occurs 2-3 weeks after completion of alcoholic fermentation. With the conversion of L-malic acid to L-lactic acid and $C0_2$, pH of wine increases, flavour compounds are produced, residual sugars are fermented and the wine becomes microbiologically stable, i.e. microbial growth stops. Little is known about the interactions between yeast and bacteria. A number of papers have been published on the production of short chain fatty acids (e.g. hexanoic, octanoic, decanoic), $S0_2$, peptides and proteins by *S. cerevisiae* and their effect on microbial growth. A few studies have shown that yeast may stimulate the growth of *O. oeni* and thereby malolactic fermentation. This is, however, ascribed to cell lysis towards the end of alcoholic fermentation [6, 8, 13, 19, 2, 25].

O. oeni is inhibited by high $S0_2$-producing yeast, but not by low $S0_2$-producing strains. It is, however, difficult to draw a clear correlation between $S0_2$ produced by yeast and bacterial inhibition, as mechanisms other than $S0_2$ may be involved.

Using aggressive washing and analytical techniques, a concentration of 3×10^s yeast cells cm^{-2} of the berry surface has been estimated. Other studies suggest a range of 10^4-10^6 cells cm^{-2}.

Saccharomyces is more commonly isolated from heavily damaged grapes. The presence of other yeast genera depends upon regional and climactic influences, the grape variety, disease pressure and level of damage of the grapes, and vineyard practices [7, 10, 12, 14, 21, 26].

In addition to stage of ripening, many factors have been identified that impact the presence and numbers of yeasts on the surface of grapes. In general, the number of yeasts present on grapes increases with ripening. Seasonal variation has also been observed with warmer and dryer years yielding increased yeast populations.

Infection with molds such as *Botrytis,* that can penetrate the berry surface, releasing nutrients, can impact the microbial flora of the surface of the grape. Infection with *Botrytis* was found to increase the numbers of yeasts by three orders of magnitude.

The insect pressure in a vineyard is also an important factor. Bees, wasps, and the fruit fly *Drosophila* have all been shown to be vectors of yeast species in vineyards. Microorganisms can adhere to the surfaces of the insects and be deposited on other fruit surfaces as the insect travels about the vineyard. As the insects are attracted to damaged fruit, they can

spread the yeasts from the surface of the damaged fruit to other sectors of the vineyard. The application of fungicides such as elemental sulfur in the vineyard may also impact the yeast species present [1, 15, 16, 22, 27, 30]. The regional climate and altitude of the vineyard can affect the yeasts found. The type of grape variety may also impact the yeast species found on the grape surface.

The factors affecting the yeasts found in fermentations are similar to those affecting the flora on the berry, such as the maturity of the fruit, age of the vineyard, variety, use of antifungal agents, climate, and vineyard location [11,16,18,19, 21,27]; Lactic acid bacteria are characterized by the production of lactic acid as a major catabolic end product from glucose.

Lactic acid bacteria can also be found on grapes, in grape must and wine, and beer. Undamaged grapes contain $<10^3$ CFU per g and the initial titer in must is low . Because of the acidic conditions (pH: 3.0-3.5) grape must provides a suitable natural habitat only for a few microbial groups which are acid tolerant such as LAB, acetic acid bacteria and yeasts. While many microbes are inhibited by ethanol concentrations above 4 vol%, ethanol tolerant species survive in young wine or wine. Besides yeasts, some *Lactobacillus* species (e.g. *Lb. hilgardii)* and *Oenococcus oeni* can grow at higher ethanol concentrations [34]. While only a few LAB species of the genera *Lactobacillus (Lb.), Leuconostoc (Lc), Pediococcus (P.), Oenococcus (O.)* and *Weissella (W.)* and the acetic acid genera *Acetobacter* and *Gluconobacter* can grow in must and wine, more than 90 yeast species have been found. Malolactic fermentation by lactic acid bacteria is occasionally desirable during vinification, but they can also produce several off-flavours in wine [3, 12, 15, 20, 21, 23, 28].

During the first days of must fermentation the CFU of LAB increases from 10^2 to 10^4-10^5 per ml. After the alcoholic fermentation and during the malic acid fermentation, the cell number can reach a titer of 10^7—10^s CFU per ml [31, 32]. The titer of different lactic acid species during alcoholic fermentation has been determined by Lonvaud-Funel. *O. oeni,* 3.4×10^6 (day 13, alcohol content: 18 vol%); *Lc. mesenteroides,* 9.6×10^4 (day 6, alcohol content: 9 vol%); *P. damnosus,* 3.8×10^4 (day 3, alcohol content: 7 vol%); *Lb. hilgardii,* 8.0×10^4 (day 3, alcohol content: 7 vol%); *Lb. brevis,* 2.0×10^4 (day 3, alcohol content: 7 vol%) and *Lb. plantarum,* 2.0×10^4 (day 3, alcohol content: 7 vol%). Lactic acid bacteria have an influence on the flavour of wine, because they can produce acetic acid, diacetyl, acetoin, 2,3- butandiol, ethyl lactate, diethyl succinate and acrolein [33]. They cause a decrease in colour up to 30%. The malolactic fermentation and the consumption of nutrients (hexoses and pentoses) as well as the production of bacteriocines [1, 6, 2, 19, 24, 29] by lactic acid bacteria lead to a stabilization of wine.

In Albania, some of wine producers produce wine with spontaneous fermentation and others produce wine with inoculated yeasts that are *Saccharomyces cerevisiae, Saccharomyces bayanus* or a mix between two yeasts, *Saccharomyces cerevisiae* and *Sacharomyces bayanus* [17]. In this study we have done wine from some Albanian grape varieties like Kallmet, Black Shesh, White Shesh, Pulz, and other grape varieties like Merlot and Kabernet.

The aim of this study is to evaluate the initial number of yeasts and lactic acid bacteria in these grape varieties, to compare the quantity of this microorganisms between these grape varieties, to evaluate the difference between yeasts and lactic acid bacteria and their performance during the two fermentations, (spontaneous and inoculated fermentations), and the effect of yeasts to lactic acid bacteria. Another aim is to evaluate the performance of yeasts growth during spontaneous fermentation compared with fermentation where *Saccharomyces bayanus* yeast is used for the inoculation, also to evaluate the quality of two wines and the effect of different yeast on the quality of the some Albanian wine.

Material and methods

100 kg of Kallmet, Black Shesh, White Shesh, Pulz, and other grape varieties like Merlot and Kabernet are used to produce wine, with spontanenous and inoculated fermentation with *Sacharomyces bayanus* yeast.

For this work the grape of variety Kallmet was harvested in the village of Kallmet (Lezhe) at 18°Brix. The quantity was divided in 2 lots of 50 kg each (K1 and K2). The analytical parameters analyzed to the grape must were: pH, total acidity, % of sugar content and chromatic characteristics. After the crushing each lot was treated with 3g/hL of SO_2 and the K1 lot was also added 2,5g/kg of medium toasted oak chips. Both lots were placed to macerate in cold temperature 5°C for 72 hours. After the cold maceration the K1 lot was inoculated with *Saccharomyces bayanus BC* at 20g/hL rate, while the K2 lot was left to spontaneously ferment. Both fermentations lasted 6 days and after the racking of the wine from the skins both lots were treated with 3g/hL of SO_2 and held in storage for the second fermentation to take place.

Black Shesh, White Shesh, Pulz, like Merlot and Kabernet wine are also prepared.

For microbiological analysis MRS and PDA medium are prepared, sterilized in autoclave in 121^0C for 15 minutes, and spread out in Petri dishes.

25 ml of wine from two fermentations are homogenized in 225 ml of peptone water. 5 tubes are filled with 9ml of peptone water and 1ml from the homogenized wine is putted in the first tube. 1 ml from the first tube is putted in the second tube. In this manner until in the 5-th tube and finally 5 dilutions are prepared, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. 1 ml from each tube is putted in Petri dishes with MRS and PDA medium. Petri dishes with MRS medium are incubated in thermostat at 30^0C. Petri dishes with PDA medium are incubated in thermostat at 25^0C. After 48-72 hour Petri dishes are taken off from the thermostat and lactic acid bacteria colonies are counted.

Results and Discussions

The results of our analysis of Kallmet, Black Shesh, White Shesh, Pulz, Merlot and Kabernet grape variety must at the first day of fermentation for yeasts' initial number are as shown in Figure 1:

Kallmet, Black Shesh, White Shesh, Pulz are autochthonous Albanian grape varieties.

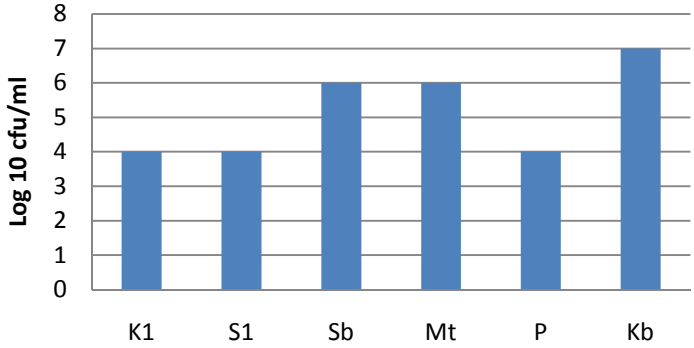

Figure 1. The initial number of yeast of of Kallmet, Black Shesh, White Shesh, Pulz, Merlot and Kabernet grape varieties.

The high number of yeast at the first day of fermentation is at Kabernet variety (1.6 x 10^7 cfu/ml), and the low initial number of yeast is at of Kallmet, Black Shesh, and Pulz, variety (~10^4 cfu/ml).The results of our analysis of Kallmet, Black Shesh, White Shesh, Pulz, Merlot and Kabernet grape variety must at the first day of fermentation for lactic acid bacteria's initial number are as shown in figure 2:

The high number of lactic acid bacteria at the first day of fermentation is at Kabernet variety (2 x 10^7 cfu/ml), and the low initial number of yeast is at of Black Shesh variety (~10^3 cfu/ml).

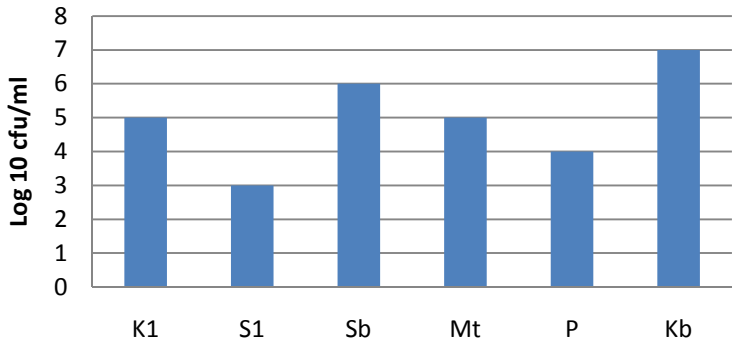

Figure 2. The initial number of lactic acid bacteria of Kallmet, Black Shesh, White Shesh, Pulz, Merlot and Kabernet grape varieties.

Figure 3. The difference between initial number of lactic acid bacteria and yeast of Kallmet, Black Shesh, White Shesh, Pulz, Merlot and Kabernet grape varieties.

The difference between initial number of lactic acid bacteria and yeast is higher at Kallmet, Black Shesh and Merlot variety. But the change is that at Kallmet variety the initial number of lactic acid bacteria is higher than yeasts and at Black Shesh and Merlot variety the initial number of yeast is higher than lactic acid bacteria.

Conclusions

In the first days of vinification for alcholic fermentation the number of yeast should be higher than the number of lactic acid bacteria. After acoholic fermentation yeasts do the autolize of their cell and can release peptide, aminoacides that can be used by lactic acid bacteria like nutrients. And in this time the growth of lactic acid bacteria can occur and also malolactic fermentation by them.

From this analysis we can say that for Black Shesh and Merlot variety we may use spontanious fermentation but for Kallmet variety is better to use inoculated fermentation with selected yeast.

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