

## NATURAL MICROFLORA OF WINE GRAPE BERRIES

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### ABSTRACT

The diversity of yeasts and bacterial species on grapes has been investigated in vineyards worldwide. For winemaking are very important three groups of microorganisms. First two includes acetic acid and lactic acid bacteria; they live and grow normally on grape surface. The third group includes more than 20 detectable genera of yeasts. There are three principal genera found on grapes *Hanseniaspora uvarum* (*Kloeckera apiculata*), *Metschnikowia pulcherrima* (*Candida pulcherrima*), and *Candida stellata* or new described *Candida zemplinina*. Aim of this study was investigate of number of three major groups of microorganisms which are important for grapes and winemaking. The number of bacteria on Acetobacter agar (AA) ranged from 1.76 log CFU/mL to 2.80 log CFU/mL. Lactic acid bacteria were counted on MRS agar and the number of detectable colonies ranged from 0.48 log CFU/mL to 2.06 log CFU/mL. Sabouraud dextrose agar (SDA) was used for cultivation of yeast and the number of yeasts ranged from 2.47 log CFU/mL to 2.76 log CFU/mL. For identification of yeast species were used different types of agar media with acid base indicator bromocresol green. Identified 10 yeasts species includes to genus: *Candida*, *Metschnikowia*, *Pichia*, *Kluyveromyces*, *Hanseniaspora*, *Hansenula*, *Candida*, *Debaromyces*, *Rhodotorula* and *Saccharomyces*. We identified only few bacterial species includes to genus *Lactobacillus*, *Pediococcus*, *Gluconobacter* and *Acetobacter*.

**Keywords:** Grapes, identification, yeasts, bacteria

### INTRODUCTION

Numerous studies have analyzed the presence of yeast on the surface of grapes (de Andrés-de Prado *et al.*, 2007; Fleet, 1990) and many have indicated that *Saccharomyces cerevisiae* is present only in very small numbers on healthy grapes (Martini, 1993; Pretorius, 2000). The most frequently isolated native species is *Kloeckera apiculata*, which may account for more than 50% of the total yeast flora recovered from fruit. Lesser numbers of other yeasts, such as species of *Candida*, *Cryptococcus*, *Debaryomyces*, *Hansenula*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, and *Rhodotorula*, have also been reported (Fleet, 1990; Velázquez *et al.*, 1991; Sabate *et al.*, 2002). The grape microflora, however, varies greatly depending on factors such as geographical location, rainfall and temperature (Longo *et al.*, 1991; Parrish and Carroll, 1985), fungicide use soil type, vineyard age, grape variety and harvesting method (Pretorius *et al.*, 1999; Rosini *et al.*, 1982). Fermentative yeasts include those responsible for wine fermentation, where *S. cerevisiae* is the most important, but other species (*S. bayanus*, *S. pastorianus* and *S. paradoxus*) may also conduct or participate in the process (Josepa, 2000; Arroyo López *et al.*, 2010). *Saccharomyces* can be found in grape musts, but the populations are often less than 50 CFU/mL (König *et al.*, 2009).

Failure to routinely isolate *Saccharomyces* from the vineyard could reflect the preference of this yeast for the high-sugar environments of grape juice and fermentation. However, the use of direct isolation techniques showed a much different picture, revealing that *Saccharomyces* spp. is an absent or rarely found on grapes (Davenport, 1974). Concerning yeasts, the innocent group includes basidiomycetous species which are regarded as irrelevant to winemaking due to their inability to ferment juice sugars or to survive in wines. The ascomycetous dimorphic fungus, *Aureobasidium pullulans* (also called black yeast), a common yeast-like species, is also technologically irrelevant. The oxidative, weakly fermentative or fermentative ascomycetous species (*Candida* spp., *Kloeckera apiculata* / *Hanseniaspora uvarum*, *Metschnikowia* spp., *Pichia* spp.) are present in pre-fermentation steps or at the beginning of fermentation. The fermentation ability is not a well-defined taxonomic feature and several species may be regarded as weakly fermentative or not (Romano *et al.*, 1997).

Like yeasts, lactic acid bacteria are also present in vineyards. Species that have been isolated from grapes include *Lactobacillus hilgardii*, *L. plantarum*, *L. casei*, *O. oeni*, *Leuconostoc mesenteroides*, and *P. damnosus* (Lonvaud-Funel *et al.*, 1999). Acetic acid bacteria are commonly associated with grapes and are normally present in wine must. Unspoiled grapes are reported to have 10<sup>2</sup> - 10<sup>3</sup> CFU/g, whereas spoiled and damaged grape can contain more than 10<sup>6</sup> CFU/g (Bartowsky, 2009). Where mold growth, particularly *Botrytis cinerea*, and grape damage develop increase of populations and diversity of acetic acid bacteria not only *G. oxydans* but *A. aceti* or *A. pasteurianus*. In agreement, González *et al.* (2006) reported a major presence of both *G. oxydans* and *A. aceti* in spoiled grapes.

Concerning bacterial species, acetic acid bacteria are regarded as innocent because they are easily controllable in the winery, although the exaggerated production of acetic acid during grape sour rot is a serious threat to wine quality. The physiological diversity of lactic acid bacteria does not allow a precise assessment of their technological significance. The typical agent of malolactic fermentation is *O. oeni* while *Lactobacillus* spp. and *Pediococcus* spp. may be responsible for spontaneous fermentations (Lerm *et al.*, 2010). These species may spoil wine when their activity goes beyond malic conversion, particularly in high pH wines, producing off flavors or biogenic amines (Arena *et al.*, 2011; Capozzi *et al.*, 2011; Pan *et al.*, 2011). Grapes also bear a wide diversity of bacterial species common in nature or in other food related environments.

Understanding microbial ecology during vinification is further complicated by mounting evidence that microorganisms can also exist in a state known as "viable but non culturable" (VBNC). By definition, microorganisms in the VBNC state fail to grow on microbiological media yet display low levels of metabolic activity (Oliver, 2005).

Microorganisms found in wine and believed to be able to enter a VBNC state are *Acetobacter aceti*, *Brettanomyces bruxellensis*, *Candida stellata*, *Lactobacillus plantarum*, *Saccharomyces cerevisiae*, and *Zygosaccharomyces bailii* (Millet and Lonvaud-Funel, 2000; Divol and Lonvaud-Funel, 2005; Du Toit *et al.*, 2005; Oliver, 2005).

The objectives of this study were to investigate the occurrence of natural microflora of grape berries in four different varieties of wine grape berries from

Nižné Valice village. Identification of three groups of microorganisms includes yeasts, lactic acid bacteria and acetic acid bacteria on grape berries with classical microbiological methods.

**MATERIAL AND METHODS**

**Collection of grape berries**

Grape berries were collected nearby Nižné Valice village. We collected four different varieties of wine grape berries, five times from one variety aseptically with gloves. The wine berries were collected from wine varieties: Blaufränkisch 2014, Cabernet Sauvignon 2014, Rhein Riesling 2014 and Welschriesling 2014. Ten berries from each variety was putted into the 50 mL centrifuge tubes (five times from each grape variety) with 25 mL of physiological saline, and stored at dark cold place for next analysis (20 samples).

**Determination of CFU and cultivation media**

For microbiological analysis the grape samples in physiological saline were processed to 24 hour after collection. For classical microbiological method - plate count method was use three specific cultivation media. We used Sabouraud dextrose agar (SDA, Conda Spain) for yeast, Acetobacter agar (glucose) (AA, HiMedia, India) for *Acetobacter* species and MRS (Conda, Spain) agar medium for lactic acid bacteria. Plate diluting method was applied for quantitative CFU (Colony Forming Units) counts determination of respective groups of microorganisms in 100 µL of bacterial solution, which inoculated into the agar medium surface. Petri dishes of gelatinous nutritive substrate were inoculated with 100 µL of bacterial solution from grape surface in three replications. Ten grape berries was homogenized and stored in 25 mL of physiological saline per one sample. Basic dilution ( $10^{-1}$   $10^{-2}$   $10^{-3}$ ) were prepared from the stock solution as follows: 0.5 mL of sample was added to the tube which contains 4.5 mL of physiological saline (0.85%). For microorganisms' cultivation were used three types of cultivation media, to segregate individual microorganism groups. SDA was used for CFU segregation of yeasts (incubation 5 days at 25°C, aerobic cultivation method), AA (incubation 48 h at 28°C), MRS (incubation 72 h at 37°C microaerophilic cultivation method). After incubation time collect the results and starts with cleaning and identification of different microbial species. For cleaning and better identification of yeasts isolated from grapes used different media: Malt Extract agar (MEA, Biomark, India) and Glucose Yeast extract agar (GYA, Conda, Spain) with indicator bromocresol green (0.020g/L). The cleaned bacterial culture was further identified by Gram staining under fluorescent microscope, and with indicator bromocresol green.

**RESULTS AND DISCUSSION**

Grape skin was colonized by different species of bacteria and yeast. The most important microorganisms for alcoholic fermentation are yeasts. Table 1 shows the results from plate diluting method at log CFU/mL unit. The number of bacteria on *Acetobacter* agar (AA) ranged from 1.76 log CFU/mL to 2.80 log CFU/mL. The highest level of acetic acid bacteria on AA was found in grape variety Blaufränkisch (2.80 log CFU/mL). Lactic acid bacteria were counted on MRS agar and the number of detectable colonies ranged from 0.48 log CFU/mL to 2.06 log CFU/mL, but negative results has been obtained from white grape varieties, when lactic acid bacteria doesn't grow. The highest level of lactic acid bacteria was found in grape variety Cabernet Sauvignon 2.06 log CFU/mL, and the highest level of lactic acid bacteria was also found in Blaufränkisch grape variety at second sample. Sabouraud dextrose agar (SDA) was used for cultivation of yeast and the number of yeasts ranged from 2.47 log CFU/mL to 2.76 log CFU/mL, and the highest level of yeasts grown up on Blaufränkisch grape variety surface.



**Figure 1** Acid producing yeast on MEA with Bromocresol green

**Table 1** Number of microorganisms isolated from grape berries in log CFU/mL

| Grape variety           | Sample | AA   | MRS  | SDA  |
|-------------------------|--------|------|------|------|
| Blaufränkisch 2014      | 1      | 2.35 | 1.20 | 2.72 |
|                         | 2      | 2.57 | 1.67 | 2.73 |
|                         | 3      | 1.94 | 1.48 | 2.72 |
|                         | 4      | 2.59 | 0.48 | 2.76 |
|                         | 5      | 2.40 | 0.48 | 2.74 |
| Cabernet Sauvignon 2014 | 1      | 1.76 | 1.70 | 2.72 |
|                         | 2      | 2.73 | 1.66 | 2.73 |
|                         | 3      | 2.29 | 1.51 | 2.73 |
|                         | 4      | 2.72 | 1.45 | 2.74 |
|                         | 5      | 2.71 | 2.06 | 2.74 |
| Rhein Riesling 2014     | 1      | 2.75 | ND   | 2.72 |
|                         | 2      | 2.71 | ND   | 2.71 |
|                         | 3      | 2.54 | ND   | 2.52 |
|                         | 4      | 2.61 | ND   | 2.55 |
|                         | 5      | 2.63 | ND   | 2.51 |
| Welschriesling 2014     | 1      | 2.53 | ND   | 2.54 |
|                         | 2      | 2.67 | ND   | 2.52 |
|                         | 3      | 2.80 | ND   | 2.47 |
|                         | 4      | 2.49 | ND   | 2.68 |
|                         | 5      | 2.29 | ND   | 2.49 |

AA - *Acetobacter* spp., MRS - *Lactobacillus* spp., SDA - Yeasts, ND - not detected Unit - log CFU/mL

For identification of yeast species was used different types of agar media with acid base indicator bromocresol green. Visual interval of acid base indicator bromocresol green is varying from pH 3.8 (yellow) to 5.4 (blue). Green color is visible at pH 4.5 (Ibarra et al., 2004).

As seen above in table 1 the highest level of grown cultures were detected in AA and SDA agar media. AA was suitable for growing yeasts and also some yeast species grown up on MRS agar. When used the pH indicator bromocresol green the acid production strains were yellow or green and strains with negative or poor acid production were white, turquoise or blue. With the production of acids agar discolored to yellow under the yeasts or bacterial colonies. Table 2 shows all discovered species coloration isolated from grapes, which cultivated on different type of agar media with bromocresol green (BG) indicator. Generally, very few yeast species ( $10^3$ - $10^6$  CFU/mL) are detected on immature grape berries, but they increase to populations of  $10^4$ - $10^6$  CFU/mL as the grapes mature to harvest. During ripening, sugars leach or diffuse from the inner tissues of the grape to the surface, thereby encouraging yeast growth. Unripe grapes harbor a predominance of *Rhodotorula*, *Cryptococcus* and *Candida* species. Most of these species are also isolated from mature, ripe grapes but, at this stage, species of the apiculate yeasts, *Hanseniaspora* (anamorph *Kloeckera*) and *Metschnikowia*, are mostly predominant. Damaged grapes have increased incidence of *Hanseniaspora* (*Kloeckera*), *Candida* and *Metschnikowia* species, as well as species of *Saccharomyces* and *Zygosaccharomyces* (Fleet, 2003).

**Table 2** Positive or negative results of agar decolorization with indicator Bromocresol green

| Grape variety           | No. of strain | Strain Color   | GYA + BG | MEA + BG |
|-------------------------|---------------|----------------|----------|----------|
| Blaufränkisch 2014      | 1.            | mint           | ±        | -        |
|                         | 2.            | blue           | +        | +        |
|                         | 3.            | green          | +        | -        |
|                         | 4.            | cherry         | ±        | -        |
| Cabernet Sauvignon 2014 | 1.            | mint           | -        | -        |
|                         | 2.            | white          | +        | -        |
|                         | 3.            | translucent    | +        | -        |
|                         | 4.            | translucent II | +        | -        |
|                         | 5.            | dark blue      | +        | -        |
|                         | 6.            | purple         | +        | -        |
| Rhein Riesling 2014     | 1.            | mint           | +        | -        |
|                         | 2.            | turquoise      | +        | -        |
|                         | 3.            | blue           | +        | -        |
|                         | 4.            | purple         | +        | -        |
|                         | 5.            | orange         | +        | -        |
|                         | 6.            | translucent    | +        | -        |
| Welschriesling 2014     | 1.            | cherry         | ±        | -        |
|                         | 2.            | purple         | +        | -        |
|                         | 3.            | translucent    | +        | -        |
|                         | 4.            | green          | +        | -        |

Agar decoloration: + positive, - negative, ± between, GYA (Glucose yeast extract agar), MEA (Malt extract agar)

Although all isolated strains grown very well on MEA + BG and on GYA + BG, but the decolorization of agar media was better on GYA + BG. Isolated microorganisms from grape berries changed the GYA medium coloration after 2-3 days of cultivating. The bromocresol green was absorbed by microorganisms'

strains. However next medium MEA with BG shows a negative results, but the strains were colorized by bromocresol green. In one case was the agar changes his color from deep green-blue to yellow (figure 1). This strain was produce high concentration of acids and the indicator was changed from blue to yellow. This positive result was detected at strain number two isolated from Blaufränkisch

grape variety. The next table shows the differences between initially described strains on MEA + BG and strains after inoculation to new agar plates with BG. Table 3 shows the colorization of mainly yeasts and bacterial strains.

**Table 3** Colorization of Yeast species by using Bromocresol green as an indicator

| Grape variety                  | No. of strain | First description | GYA + BG                   | MEA + BG          |
|--------------------------------|---------------|-------------------|----------------------------|-------------------|
| <b>Blaufränkisch 2014</b>      | 1             | mint              | green + turquoise          | mint              |
|                                | 2             | blue              | green                      | transcurent       |
|                                | 3             | green             | white + dark green         | mint + green      |
|                                | 4             | cherry            | pinky dark green           | cherry            |
| <b>Cabernet Sauvignon 2014</b> | 1             | mint              | mint + dark green          | mint              |
|                                | 2             | white             | white                      | mint              |
|                                | 3             | transcurent       | green                      | transcurent       |
|                                | 4             | transcurent II    | dark green                 | transcurent       |
|                                | 5             | dark blue         | yellow green               | dark blue         |
|                                | 6             | purple            | yellow green               | purple            |
| <b>Rhein Riesling 2014</b>     | 1             | mint              | mint + green               | mint              |
|                                | 2             | turquoise         | green + turquoise          | white             |
|                                | 3             | blue              | green + turquoise          | mint              |
|                                | 4             | purple            | pink + dark green          | purple            |
|                                | 5             | orange            | dark green                 | cherry            |
|                                | 6             | transcurent       | green                      | transcurent       |
| <b>Welschriesling 2014</b>     | 1             | cherry            | pink + dark green          | cherry            |
|                                | 2             | purple            | pink + dark green + purple | purple            |
|                                | 3             | transcurent       | green                      | transcurent       |
|                                | 4             | green             | dark green + mint          | dark green + mint |

First description on MEA + BG, GYA (Glucose yeast extract agar), MEA (Malt extract agar), BG (Bromocresol green).

We compared the obtained descriptions by using microscope with the descriptions from other authors. Many species were isolated from grape berries. These yeast and bacteria species are includes to natural microflora of grape berries (König et al., 2009).

Numerous yeast genera and species are found during the production of wine. The low pH of wine, high sugar content, rapidly generated anaerobic conditions, and presence of phenolic compounds creates the ideal environment to support the growth of yeasts and to enrich these organisms over other microbes (Fleet, 2003; Romano et al., 1997).

The yeasts that impact the composition of the wine can come in with the grapes from the vineyard, can be residents of the winery flora, or can be spread by insect vectors such as fruit flies, bees, and wasps (Fleet et al., 2002). Over twenty yeast genera have been identified from wines (Renouf et al., 2007).

There are three principal genera found on grapes: *Hanseniaspora uvarum* (anamorph: *Kloeckera apiculata*), *Metschnikowia pulcherrima* (anamorph: *Candida pulcherrima*), and *Candida stellata*. In some reports, *Hanseniaspora* is the dominant species (Beltran et al., 2002; Combina et al., 2005; Hierro et al.,

2006) and in others it is *Candida* (Clemente-Jimenez et al., 2004; Torija et al., 2001). *Candida* has been shown to complete the alcoholic fermentation in some cases (Clemente-Jimenez et al., 2004). Several of the *Candida stellata* isolates from wine are actually *Candida zemplinina* (Csoma and Sipiczki, 2008).

Other yeasts can be commonly found, although they are not as universal. *Saccharomyces* can be detected, but is present on grape surfaces at very low levels (Prakitchaiwattana et al., 2004; Martini et al., 1996), and has been undetectable in some studies (Combina et al., 2005; Raspor et al., 2006).

We isolated and determined 10 different species of yeasts, two *Lactobacillus* and *Acetobacter* strains and one *Pediococcus*, *Gluconobacter* strain. The most dominant species in this study were *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Hansenula anomala*, *Kluyveromyces marxianus* and *Candida stellata*. We also isolated *Pichia*, *Debaryomyces*, *Dekkera* and *Saccharomyces* yeasts from grape surfaces. Table 4 shows the results from determined and detected species on grape berries.

**Table 4** Isolated microorganisms from grape berries

| Grape variety                  | No. of strain | Yeast   | Bacteria                 | Reference                        |
|--------------------------------|---------------|---|--------------------------|----------------------------------|
| <b>Blaufränkisch 2014</b>      | 1             | <i>Candida</i> sp.                                      | ND                       | Konig et al., 2009               |
|                                | 2             | <i>Dekkera bruxellensis</i>                             | ND                       | Konig et al., 2009               |
|                                | 3             | <i>Hansenula anomala</i> , <i>Debaryomyces hansenii</i> | <i>Lactobacillus</i> sp. | Fleet, 2003, Andriy et al., 2009 |
|                                | 4             | <i>Metschnikowia pulcherrima</i>                        | <i>Acetobacter</i> sp.   | Fernández et al., 2000           |
| <b>Cabernet Sauvignon 2014</b> | 1             | <i>Pichia anomala</i>                                   | ND                       | Fleet, 2003                      |
|                                | 2             | <i>Hansenula anomala</i>                                | ND                       | Fleet, 2003                      |
|                                | 3             | <i>Dekkera bruxellensis</i>                             | <i>Gluconobacter</i> sp. | Konig et al., 2009               |
|                                | 4             | <i>Hanseniaspora uvarum</i>                             | <i>Lactobacillus</i> sp. | Delfini et al., 2002             |
|                                | 5             | <i>Saccharomyces</i> sp., <i>Pichia</i> sp.             | <i>Pediococcus</i> sp.   | Konig et al., 2009               |
|                                | 6             | <i>Kluyveromyces marxianus</i>                          | ND                       | Kourkoutas et al., 2002          |
| <b>Rhein Riesling 2014</b>     | 1             | <i>Pichia anomala</i>                                   | ND                       | Fleet, 2003                      |
|                                | 2             | <i>Hansenula anomala</i>                                | ND                       | Fleet, 2003                      |
|                                | 3             | <i>Candida</i> sp.                                      | ND                       | Konig et al., 2009               |
|                                | 4             | <i>Kluyveromyces marxianus</i>                          | ND                       | Kourkoutas et al., 2002          |
|                                | 5             | <i>Rhodotorula mucilaginosa</i>                         | <i>Acetobacter</i> sp.   | Quesada et al., 1995             |
|                                | 6             | <i>Hanseniaspora uvarum</i>                             | ND                       | Delfini et al., 2002             |
| <b>Welschriesling 2014</b>     | 1             | <i>Rhodotorula mucilaginosa</i>                         | ND                       | Quesada et al., 1995             |
|                                | 2             | <i>Kluyveromyces marxianus</i>                          | ND                       | Kourkoutas et al., 2002          |
|                                | 3             | <i>Hanseniaspora uvarum</i>                             | ND                       | Delfini et al., 2002             |
|                                | 4             | <i>Metschnikowia pulcherrima</i>                        | ND                       | Fernández et al., 2000           |

ND - Not detected



## CONCLUSION

Natural microflora of grape berries is very diverse. After plate diluting method shows us that the most various microorganisms on grape berry surface are yeasts. More lactic acid bacteria colonies grow up from blue grape varieties Blaufränkisch 2014 and Cabernet Sauvignon 2014 cultivated on MRS, but on white varieties Rhein Riesling 2014 and Welschriesling 2014 doesn't grow up. The detection level of yeast was very similar on all tested samples of grape berries. By microscopic determination identified different yeast species includes to 10 genera. We tried to cultivate acetic acid bacteria on Acetobacter agar, but as shows the results sooner, yeast grow very well on this agar, and after gram staining and microscopic determination we isolated only 2 *Acetobacter* and one *Gluconobacter* strain. We detected on grape berries only three strains of lactic acid bacteria.

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