

EVALUATION OF LACTIC ACID BACTERIA GROWTH DURING AUTOCHTHONOUS ALBANIAN KALLMET WINE PRODUCTION WITH SPONTANEOUS AND INOCULATED FERMENTATIONS

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Abstract: The grape used in wine making has many wild microorganisms like lactic acid bacteria, yeast, acetic acid bacteria. During the alcoholic fermentation, the evaluation of these microorganisms depends on their activity. There is an interaction between yeast and lactic acid bacteria during this period of wine making. In this study, we have made wine from the autochthonous Albanian grape Kallmet variety using the spontaneous fermentation and inoculated fermentation with the yeast *Saccharomyces bayannus*. Yeasts carry out the alcohol fermentation, and lactic acid bacteria make malolactic fermentation in wine. With this fermentation, lactic acid bacteria convert malic acid to lactic acid, reducing the acidity of the wine and create a microbiological stability. During the alcoholic fermentation, the evaluation of lactic acid bacteria is not required. The aim of our study is to evaluate the first quantity of lactic acid bacteria to Kallmet grape, their performance during the two fermentations, spontaneous and inoculated fermentations.

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Introduction

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 *Saccharomyces bayanus* (Hasalliu, 2016).

Must has a low pH value (3-3.5) as well as a high sugar concentration. Therefore, it represents a very specific habitat in which the chemical and physiological conditions are changing during vinification. There are only three groups of microorganisms that are adapted to these harsh conditions: yeasts, acetic acid bacteria (AAB) and lactic acid bacteria (LAB). These microbes also influence the wine making process itself (König et al., 2009, Petri et al., 2013).

Lactic acid bacteria can already be found on grapes. However, undamaged grapes contain less than 10^3 colony-forming units (CFU)/g leading to a low initial titer in must (Lafon-Lafourcade et al., 1983). Only a few LAB species of the genera *Lactobacillus* (Lb.), *Leuconostoc* (Lc), *Pediococcus* (P.), *Oenococcus* (O.) and *Weissella* (W.) can grow in must, whereas carnobacteria, enterococci, lactococci, streptococci, and bifidobacteria have not been isolated from must or wine. The acetic acid bacteria *Acetobacter* and *Gluconobacter* as well as more than 90 yeast species have also been found. Yeasts convert glucose of the sugar-rich must into alcohol. Most of the yeasts will usually die out once the alcohol level reaches about 5 %, whereas more alcohol tolerant *Saccharomyces* species take over. Also, the CFU of LAB decreases after an initial increase from 10^2 to 10^4 - 10^5 CFU/ml during the first days of must fermentation. During alcoholic fermentation, LAB do not multiply or even disappear, except *O. oeni*, which resists at low cell levels.

We studied this growth in our experiments with the Albanian grape variety Kallmet.

After alcoholic fermentation, the growth of this species is stimulated because of the released cell constituents of yeasts, which are in a stationary or death phase. At this stage, oenococci have an influence on yeast lysis by producing glycosidases and proteases (König et al., 2009)

Only some other ethanol tolerant LAB species survive in young wine or wine. Furthermore, it was found that fatty acids (hexanoic, octanoic and decanoic acid) liberated by growing yeast also have a negative effect on bacterial growth (Lonvaud-Funel et al., 1991; Hui, 1995).

Relevance of LAB in wine and malolactic fermentation by LAB

LAB plays an important role in wine quality. The malolactic fermentation carried out by these bacteria leads to de-acidification and stabilization of wines. LAB converts malate into lactate and CO_2 after alcoholic fermentation. This so called malolactic fermentation primarily leads to biological de-acidification, but also to flavor modifications and microbial stabilization. Therefore, it has a positive effect on wine quality (Liu, 2002).

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In addition to malate, lactic acid bacteria also metabolize tartrate and citrate, which also belong to the main acids of must. Citrate is metabolized to lactate, acetic acid, CO₂, and acetoin. According to Radler and Yannissis (1972), tartrate can be converted to lactate, acetate, and CO₂ by homofermentative LAB (e.g., *Lb. plantarum*) and to acetate and CO₂ or fumaric acid (succinic acid) by heterofermentative LAB (e.g. *Lb. brevis*) (Llauberes et al, 1990; Duenas et al, 2003).

Even though malolactic fermentation can occur spontaneously, *O. oeni* is often used as a bacterial starter culture. Because of its high tolerance to ethanol and acidity, this species is the preferred starter culture applied for the reduction of the malic acid content (Brandolini et al., 2002). Especially in northern countries, where must can have a high acidity, the biological reduction with such starter cultures is an important step in vinification. However, the malolactic enzyme has also been found in many other lactic acid bacteria occurring in wine (e.g., *Lb. casei*, *Lb. brevis*, *Lb. buchneri*, *Lb. delbrueckii*, *Lb. hilgardii*, *Lb. plantarum* and *Lc. mesenteroides*) (Schiitz and Radler, 1984).

The malolactic fermentation and the consumption of nutrients (hexoses and pentoses), as well as the production of antimicrobial components (De Vuyst and Vandamme, 1994), can lead to more stable wines. Lactic acid bacteria potentially produce acetic acid, higher concentrations of CO₂, H₂O₂, diacetyl, pyroglutamic acid and bacteriocins, which inhibit the growth of other bacterial and yeast species (Rammelberg and Radler, 1990; Blom and Mortvedt, 1991). For example, it is known that the bacteriocin brevicin from *Lb. brevis* inhibits the growth of *O. oeni* and *P. damnosus* (Rammelberg and Radler, 1990).

Spoilage by LAB (e.g., mousiness, ropiness)

In Albania, we do not have studies about the growth and the effects of lactic acid bacteria in wine.

Nevertheless, the role of LAB in winemaking is ambivalent as this fermentation is only occasionally desirable during vinification in some wines. Exopolysaccharide production leads to graisse of the must, which causes problems during filtration. Different strains of *P. damnosus*, *P. parvulus* or *Leuconostoc mesenteroides* are well known for the formation of exopolysaccharide slimes, which lead to so called ropy wines (Vincenzini et al., 2009; König et al., 2009). Viscosity, which is influenced by many factors such as the ethanol concentration and temperature, becomes apparent at 10⁷ CFU/ml. Especially *P. damnosus* increases the viscosity by producing a glucose homopolymer.

Lactobacilli can also cause a loss of color (up to 30 %) after malolactic fermentation. This is due to pH changes that cause a shift in the equilibrium of anthocyanins, which contribute to the stability of color in wine.

Several LAB are also involved in the generation of numerous off-flavors, because they can produce acetic acid, diacetyl, acetoin, 2,3-butanediol, ethyl lactate, diethyl succinate, acrolein, mannitol and compounds that form the geranium note or mousy-off flavor (König et al., 2009; Petri et al., 2013).

Mousy-off flavor or mousiness is a smell reminiscent of mice. Lactobacilli such as *Lb. brevis*, *Lb. hilgardii* and *Lb. fermentum* produce acetyl-tetrahydropyridine (perception threshold: 1.6 ng/l) from ethanol and lysine. Acetyl-pyrroline and ethyl-tetrahydropyridine can also contribute to this off-flavor (Vincenzini et al., 2009; Nakayama and Sonomoto, 2002).

Diacetyl and acetoin can be formed by the metabolism of citrate if the excess of pyruvate is reduced to lactic acid.

Fructose is reduced to mannitol or converted to erythritol and acetate. When *Lb. plantarum* is grown on mannitol, oxaloacetate can also function as electron acceptor leading to the formation of succinic acid (Chen and McFeeters, 1986).

Glycerol is converted to propanediol and acrolein leading to bitterness (Schiitz and Radler, 1984).

O. oeni can also produce off-flavors due to the metabolism of cysteine and methionine. Hence, cysteine is transformed into hydrogen sulfide or sulfanyl ethanol and methionine into dimethyl disulfide, propanol, and propionic acid. The latter has an earthy, red-berry fruit flavor (Ribereau-Gayon et al., 2006).

Health hazards due to LAB (e.g., biogenic amines, ethylcarbamate)

Biogenic amine production (e.g., tyramine, histamine or putrescine), is also mainly caused by microbiological activities of some LAB strains during vinification. *O. oeni*, *P. cerevisiae*, and *Lb.*

higardii are examples of producers of biogenic amines (Landete et al., 2005; Mangani et al., 2005; Sebastian et al., 2011; Petri et al., 2013).

The most important biogenic amine is histamine, which is produced by decarboxylation of histidine. The COST Action 917 (2000-2001) of the EU "Biologically active amines in food" suggested prescriptive limits for histamine (e.g., France: 8 mg/l, Germany: 2 mg/l) in wines. Biogenic amines can cause health problems like migraine and head ache (Coton et al., 1998) and sensory defects in wine (Lehtonen, 1996). From arginine, ammonium is liberated by heterofermentative species such as *Lb. higardii* and *O. oeni*, but also by facultatively heterofermentative species like *Lb. plantarum*.

Ethyl carbamate, which is probably carcinogenic, is produced from urea and ethanol by *O. oeni* and *Lb. higardii* (Uthurry et al., 2006). Alcoholic fermentation must prevail against malolactic fermentation in the first days of fermentation. The growth of lactic acid bacteria in the first days of fermentation may cause shortcomings of the wine.

The aim of our study is to evaluate the first quantity of lactic acid bacteria to Kallmet grape, their performance during the two fermentations, (spontaneous and inoculated fermentations).

Materials and methods

100 kg of Kallmet grape (Figure 1) were used to produce Kallmet wine with spontaneous fermentation and fermentation inoculated 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 into 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 3 g/hL of SO₂ 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 20 g/hL rate, while the K2 lot was left to ferment spontaneously. Both fermentations lasted six days and after the racking of the wine from the skins both lots were treated with 3 g/hL of SO₂ and held in storage for the second fermentation to take place.

For microbiological analysis MRS medium was prepared, sterilized in an autoclave in 121°C for 15 minutes, and spread out in Petri dishes.

Figure 1: Kallmet grape (Autochthonous Albanian grape variety)



Source: Author

25 ml of wine from two fermentations was homogenized in 225 ml of peptone water. Five tubes were filled with 9 ml of peptone water, and 1 ml from the homogenized wine was placed in the first tube, 1 ml from the first tube was placed in the second tube and in the same manner until the fifth tube. Finally, five dilutions were prepared: 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵. 1 ml from each tube was placed in Petri dishes with MRS medium. Petri dishes with MRS medium were incubated in a thermostat at 30°C. After 48-72 hours, Petri dishes were taken from the thermostat, and lactic acid bacteria colonies were counted.

Results and Discussions

The results of Kallmet wine produced in this study for total acidity were 5.5 ± 0.08 for K1 samples (inoculated fermentation with *S. bayanus*) and 6.1 ± 0.05 for K2 (spontaneous fermentation). The results for pH of the Kallmet wine were 4.12 ± 0.02 for K1 wine (inoculated fermentation with *S.*

bayanus) and 3.98 ± 0.02 for K2 wine with spontaneous fermentation). The results for the % of alcohol of Kallmet wine was 11.9 ± 0.03 for K1 wine (inoculated fermentation with *S. bayanus*) and 12 ± 0.02 for K2 wine (spontaneous fermentation).

Due to its tolerance against ethanol and acidic conditions, LAB (Lactic Acid Bacteria) can grow in must. During the first days of must fermentation, the CFU of LAB increased from 10^5 to 10^6 - 10^7 per ml (Table 1).

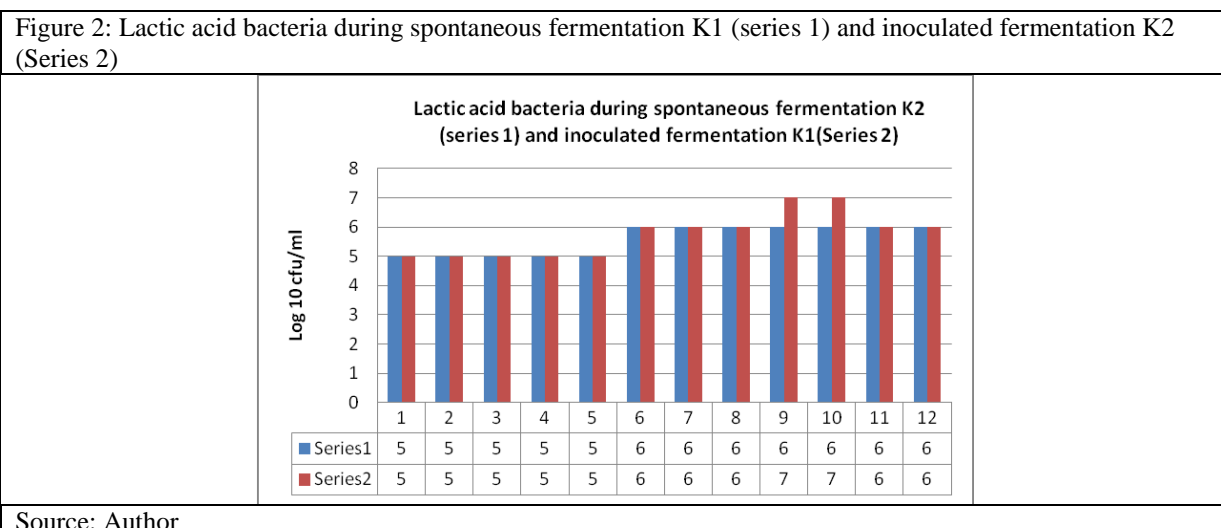
Days of fermentation	K1(Kallmet Wine inoculated with <i>S. bayanus</i>) cfu/ml	K2 (Kallmet wine with spontaneous fermentation) cfu/ml
1	2×10^5	2×10^5
6	1.1×10^6	1.7×10^6
8	7×10^6	7.1×10^6
9	8×10^6	1.8×10^7
10	2.8×10^6	2.2×10^7
11	1.2×10^6	4.7×10^6
12	6.7×10^6	1.3×10^6

Source: Author

The initial quantity of lactic acid bacteria is the same for the two samples K1 and K2 because it was the first day of fermentation and yeasts were just added and they have not started their activity yet. This quantity is the first quantity that is in Kallmet grape.

With the start of fermentation the number of lactic acid bacteria in K2 samples, (wine without inoculated yeasts) was higher (1.8×10^7 , day 9) than K1 samples (8×10^6 , day 9) of wine with inoculated yeasts *Saccharomyces bayanus*.

On the 10th day of fermentation, the number of lactic acid bacteria in K2 samples, (wine without inoculated yeasts) was also higher (2.2×10^7) than in K1 samples (2.8×10^6) of wine with inoculated yeasts *Saccharomyces bayanus* (Figure 2). This is because inoculated yeasts inhibit the growth of lactic acid bacteria. Yeasts cells are bigger than lactic acid bacteria cells, and they are competitive against lactic acid bacteria for the nutrient. Yeasts produce secondary components like toxic components that inhibit the growth of lactic acid bacteria and others spoilage bacteria for the wine like acetic acid bacteria or spoilage yeasts.



Lactic acid bacteria influence the flavor of wine because they can produce acetic acid, diacetyl, acetoin, 2,3-butanediol, ethyl lactate, diethyl succinate and acrolein.

Conclusions

Lactic acid bacteria are present in grape, must and wine, but their presence during alcoholic fermentation must not influence the activity of yeast for the normal alcoholic fermentation. The

activity and the growth of the number of lactic acid bacteria is be more desirable after alcoholic fermentation to avoid a stuck of alcoholic fermentation.

From the microbiological analyses of two samples K1 (Kallmet wine with inoculated yeasts *Saccharomyces bayanus*) and K2 (Kallmet wine without inoculated yeasts *Saccharomyces bayanus*), the initial number of lactic acid bacteria was 2×10^5 CFU/ml but during the spontaneous fermentation the number of lactic acid bacteria is higher than in wine inoculated with *S. bayanus* in days 9 and 10 of fermentations.

Inoculated yeasts *Sacharomyces bayanus*, therefore, cause the inhibition of lactic acid bacteria during the process of fermentation, during the production of Kallmet wine with autochthonous Kallmet grape.

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