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REGULAR ARTICLE

LACTIC ACID AND ACETIC ACID BACTERIA ISOLATED FROM RED WINE

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ABSTRACT

The aim of our study was the identification of red wine microbiota during the fermentation process using a classical microbiological method and real-time PCR. The changes in different groups of microorganisms were monitored in total counts of bacteria, *Lactobacillus* cells and *Acetobacter* cells. Microbiological parameters were observed during the current collection and processing of wine in 2012. Samples were taken during the fermentation process in wine enterprises and were storaged with different conditions. During this period were examined 4 bottles of wine berween Cabernet Sauvignon and Frankovka modra. The total counts of bacteria ranged from 4.98±0.08 in the wine Cabernet Sauvignon at 4 °C of storage to 5.63±0.13 log CFU.ml^{-1} in the wine Cabernet Sauvignon at 25 °C of storage. The number of lactobacilli ranged from 2.18±0.10 in the Cabernet Sauvignon at 4 °C

to 2.49±0.04 log CFU.ml^{-1} in the Frankovka modra wine at 25 °C of storage and the number of *Acetobacter* cells ranged from 4.21±0.04 in the Cabernet Sauvignon at 4 °C of storage to 4.52±0.15 log CFU.ml^{-1} in Cabernet Sauvignon at 25 °C of storage. The presence and sensitivity of Gram-positive and Gram-negative bacterial species *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus salivarius, Acetobacter aceti, Acetobacter pasteurianus* and *Acetobacter orleaniensis* were detected using Real time PCR.

Keywords: red wine, fermentation process, lactic acid bacteria, acetic acid bacteria

INTRODUCTION

The must is the first intermediate product in winemaking and characterized by low pH values and a high sugar concentration. Therefore, it represents a very specific habitat in which the chemical and physiological conditions are changing during the wine production process. There are three groups of microorganisms which are adapted to these conditions and influence the vinification itself: yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (**Petri *et al.*, 2013**).

Lactic acid bacteria (LAB) are a diverse group of Gram+, non-spore forming, catalase negative bacteria. They are chemo-organotrophic and ferment hexoses mainly to lactate (homofermentatives) or to a mixture of lactate, ethanol or acetate and CO_2 (heterofermentatives). Lactic acid bacteria are found on plants, in sewage, in the genital, intestinal and respiratory tracks of man and animals, and in fermented foods and beverages (dairy products, meat, fish, vegetables, sour dough, beer and wine). In winemaking LAB coming from grapes or from cellar machinery develop during the process showing the characteristic dynamics of the species. Some LAB are responsible for malolactic fermentation, a secondary "fermentation" which reduces acidity, gives a higher microbiological stability and improves organoleptic characteristics. LAB are also responsible for some defaults in wine such as: ropiness, lactic spoilage, mannitol taint, etc. (**Rodas *et al.*, 2003**).

The ubiquitous acetic acid bacteria (AAB) fall within the *Acetobacteraceae* family and are well adapted to various sugar and ethanol rich environments. Their ability to efficiently convert ethanol through acetaldehyde to acetic acid is utilized in culinary and medicinal vinegar production; however, in the wine industry this capability constitutes spoilage

(**Bartowsky and Henschke, 2008**). Wines spoiled by AAB have characteristic volatility, a vinegar-like sourness on the palate and a range of acetic, nutty, sherry-like, solvent or bruised apple aromas and often a reduction in fruity characters (**Bartowsky *et al*., 2003**). Such wines have low commercial value but can in some cases be improved by blending or treatment by a reverse-osmosis process to lower acetic acid content.

The objectives of this study were to investigate the occurrence of lactic acid bacteria and acetic acid bacteria in two different Slovakian red wines during fermentation process and to identify the dominant lactic acid and acetic acid bacteria strains with Real time PCR method.

MATERIAL AND METHODS

Microbiological parameters were observed during the current collection and processing of grapes in the year 2012. Samples were taken during the fermentation process in wine enterprises. During this period were examined 4 bottles of wine in week interval among two varieties of Cabernet Sauvignon and Frankovka modra. The bottles were storaged at two different temperatures first at 4 °C in refrigerator and second at 25 °C at room temperature.

Determination of CFU counts

For microbiological analysis the wine samples were processed immediately after collection. The total counts of bacteria (TBC), number of *Lactobacillus* cells (L) and number of *Acetobacter* cells (A) were assessed. Plate diluting method was applied for quantitative CFU (Colony Forming Units) counts determination of respective groups of microorganisms in 1 ml of wine. Petri dishes of gelatinous nutritive substrate were inoculated with 1 ml of wine samples (TBC, L, A) in three replications. Homogenized samples of wine were prepared in advance by sequential diluting based on decimal dilution system application. For microorganism cultivation three types of cultivating mediums were used, to segregate individual microorganism groups. Glucose Tryptone Yeats agar was used for CFU segregation of TBC (incubation 72 h at 30 °C, aerobic cultivation method). Lactobacillus MRS agar was used for CFU segregation of lactobacilli (incubation 72 h at 37 °C, microaerophilic cultivation method) and Acetobacter agar was used for CFU segregation of *Acetobacter* cells (incubation 24-48 h at 35-37 °C). Cultivating medium composition corresponded to producer introductions (Biomark^{TM}, Pune, India). Basic dilution (10^{-1}) was

prepared as follows: 5 ml of wine was added to the bank containing 45 ml of distilled water. The cells were separated from substrate in shaking machine (30 minutes). Prepared basic substance was diluted to reduce the content of microorganisms below 300 CFU level.

Bacterial Strains and DNA Extraction

For isolation of DNA, growth colonies of bacteria that we had isolated previously from individual samples in pure culture were used. Before DNA isolation of Gram-positive bacteria was prepared in peptone water the following composition: peptone 10 g, NaCl 5.0 g, distilled water 1000.0 ml. Peptone and NaCl in hot water were dissolved, filtered and pH adjusted to 7.2 to 7.8, as appropriate and then sterilized in an autoclave at 0.1 MPa for 20 minutes. For isolation of GenElue TM Bacterial Genomic DNA Kit (Sigma Aldrich, St. Louis, USA) were used:

G^+ bacteria: 1.5 ml of 24 hours bacterial culture was centrifuged during 2 min / 12.000 to 16.000 g. The supernatants were removed, the pellet was dissolved in 200 µl lysis solution and during 30 min / 37 ºC were incubated, 20 µl proteinase K were added and during 30 min / 55 ºC were incubated, then 200 µl of lysis solution C were added, about 15 s vortex mixed and at 55 ºC incubated for 10 min. We added 500 ul Column Prep. Solution to each GenMiniprepBindinb Colum, about 12 000 g centrifuged for 1 min 200 µl of ethanol (95-100 %) were added in the lysate and vortex mixed 5-10 sec. Then about 6 500 g centrifuged for 1 min. The eluates were removed, 500 µl washing buffer was added, then centrifuged at maximum speed unless drying of membrane and then transferred to a new Eppendorf tube, 200 µl of elution solution directly to the center of the membrane was added, then centrifuged for 1 min. at 6500 g.

G^- bacteria: 1.5 ml of 24 hours bacterial culture was centrifuged during 2 min / 12.000 to 16.000 g. The supernatants were removed. The pellet was dissolved in 180 µl lysis solution T, 20 µl proteinase K were added and during 30 min / 55 ºC were incubated, then 200 µl of lysis solution C were added, about 15 s vortex mixed and at 55 ºC incubated for 10 min. We added 500 ul Column Prep. Solution to each GenMiniprepBindinb Colum, about 12 000 g centrifuged for 1 min 200 µl of ethanol (95-100 %) were added in the lysate and vortex mixed 5-10 sec. Then about 6 500 g centrifuged for 1 min. The eluates were removed, 500 µl washing buffer was added, then centrifuged at maximum speed unless drying of membrane and then transferred to a new Eppendorf tube, 200 µl of elution solution directly to the center of the membrane was added, then centrifuged for 1 min. at 6 500 g.

Measuring the concentration of DNA - UV-spectrophotometric quantification of DNA (UV 1101, Biotech, UK)

The absorbance at a wavelength of 260 nm was measured and DNA concentration was calculated on base of the observation that double-stranded DNA solution with a concentration of 5 $\mu g.ml^{-1}$ has a density about 0.1. To determine the contamination of the protein preparation the additional measuring absorbance at 280 nm was used. Preparation is considered as uncontaminated if proteins A_{260}/A_{280} is between 1.8 and 2.0.

Primers used:

Acetobacter aceti: Primer name: AQ1F, AQ2R (54 bp). Steps of RTQ PCR: 5 min at 94 ºC and then cycled 35 times at 94 ºC for 1min, 58 ºC for 1min and 72 ºC for 2 min. The samples were incubated for 10 min at 72 ºC for final extension and kept at 4 ºC until tested.

Acetobacter pasteurianus: Primer name: FIP, RIP (93 bp). Steps of RTQ PCR: 5 min at 94 ºC and then cycled 35 times at 94 ºC for 1min, 58 ºC for 1min and 72 ºC for 2 min. The samples were incubated for 10 min at 72 ºC for final extension and kept at 4 ºC until tested.

Acetobacter orleaniensis: Primer name: REPIR-I, REP2-I (559 bp). Steps of RTQ PCR: 5 min at 94 ºC and then cycled 35 times at 94 ºC for 1min, 58 ºC for 1min and 72 ºC for 2 min. The samples were incubated for 10 min at 72 ºC for final extension and kept at 4 ºC until tested.

Lactobacillus salivarius: Primer name: Lsal-1, Lsal-2 (411 bp). Steps of RTQ PCR: incubation 95 ºC, 3 min., denaturation 94 ºC, 30 s., annealing 60 ºC, 60 s, extension 72 ºC 60 s., from 2^{nd} to 4^{th} step 35 cycles were repeated, the last step of the operation was temperature 72 ºC for 10 min.

Lactobacillus acidophilus: Primer name: Laci-1, 23-10C (210 bp). Steps of RTQ PCR: incubation 95 ºC 3 min., denaturation 94 ºC, 30 s., annealing 68 ºC, 60 s., extension 72 ºC, 60 s., from 2^{nd} to 4^{th} step 45 cycles were repeated, the last step of the operation was temperature 72 ºC for 10 min.

Lactobacillus crispatus: Primer name: Cri 16SI, Cri 16SII (734 bp). Steps of RTQ PCR: incubation 93 ºC, 2 min., denaturation 93 ºC, 30 s., annealing 60 ºC, 30 s. Extension 72 ºC, 30 s., from 2nd to 4th step 30 cycles were repeated, the last step of the operation was temperature 72 ºC for 10 min.

Real-time PCR

Data were collected during each elongation step. PCR products were detected by monitoring the increase in fluorescence of the reporter dye at each PCR cycle. Applied Biosystems software plots the normalized reporter signal, ΔRn, (reporter signal minus background) against the number of amplification cycles and also determines the threshold cycle (Ct) value i.e. the PCR cycle number at which fluorescence increases above a defined threshold level were used.

RESULTS AND DISCUSSION

Lactic acid bacteria (LAB) play an important role during the wine production process and have a critical impact on its quality. Therefore, the detection of these microorganisms during the vinification process is a matter of interest (**Petri *et al*., 2013**).

Acetic acid bacteria had long been believed to play little, if any, role during winemaking operations due to their aerobic nature. Winemaking in general is an anaerobic process and the growth and survival of these bacteria under this and other unfavorable conditions like high ethanol concentrations, low pH and high SO_2 concentrations seems unlikely. Some studies have, however, shown that acetic acid bacteria can survive during fermentation and the following operations in the winemaking process, such as malolactic fermentation and during maturation of the wine. Acetic acid bacteria had been shown to contribute significantly to volatile acidity in must and wine, and the production of acetic acid by these bacteria may thus also contribute to sluggish or stuck fermentations (**Du Toit and Lambrechts, 2002**).

From microbiological parameters in wine samples total counts of bacteria, number of lactobacilli and *Acetobacter* cell were monitored. We also monitored using real time polymerase (RTQ PCR) qualitative representation of individual species of microorganisms from the wine samples during fermentation. Table 1 shows the number of total counts of bacteria, number of lactobacilli and *Acetobacter* cell in Cabernet Sauvignon and Frankovka

modra samples which were collected from wine after three weeks of storage in different temperature.

The results of microorganisms number in red wine are in table 1. The total number of bacteria ranged from 4.13 to 5.77 log CFU.mL^{-1}, the number of lactobacilli ranged from 2.07 to 2.52 log CFU.mL^{-1} and the number of *Acetobacter* cells ranged from 4.18 to 4.63 log CFU.mL^{-1}. The higher number of total count of bacteria was found in Cabernet Sauvignon at 25 °C of storage. The higher number of lactobacilli was found in Farnkovka modra at 25 °C of storage and number of *Acetobacter* cells was found in Cabernet Sauvignon at 25 °C of storage. The statistical significant difference was found only between Cabernet Sauvignon at 4 °C and 25 °C of storage ($P \leq 0.05$).

Table 1 Number of microorganisms in log CFU.mL^{-1}

Type of wine		TCB	L	A
Cabernet Sauvignon at 4 °C	**1.**	4.95	2.07	4.21
	2.	4.13	2.21	4.18
	3.	5.73	2.26	4.25
Cabernet Sauvignon at 25 °C	**1.**	5.52	2.26	4.35
	2.	5.77	2.35	4.59
	3.	5.61	2.38	4.63
Frankovka modra at 4 °C	**1.**	5.39	2.52	4.52
	2.	4.88	2.48	4.59
	3.	5.16	2.39	4.25
Frankovka modra at 25 °C	**1.**	5.38	2.45	4.50
	2.	5.74	2.52	4.25
	3.	5.15	2.51	4.63

TCB-total count of bacteria, L-lactobacilli, A-*Acetobacte*r cells

In the study **Kačániová *et al.* 2012**, the total number of bacteria in Cabernet Sauvignon ranged from 0 in young wine to 92 CFU.ml^{-1} in fermentation and the number of lactobacilli ranged from 0 in young wine to 92 CFU.ml^{-1} in fermentation were found.

On some occasions, during industrial wine-making, the development of lactic acid bacteria and MLF are unpredictable, since this can occur during alcoholic fermentation or even during storage or ageing. In these cases, as a consequence of the metabolism of these bacteria, changes occur in the wine composition that can alter its quality, in some cases producing a product which is unacceptable for consumption. These alterations include the so-called ‘‘lactic disease’’, the production of undesirable aromas due to the formation of volatile phenols or aromatic heterocyclic substrates (**Costello and Henschke, 2002**), and the

production of biogenic amines (**Landete *et al.*, 2005; Marcobal *et al.*, 2006; Moreno-Arribas *et al.*, 2000**).

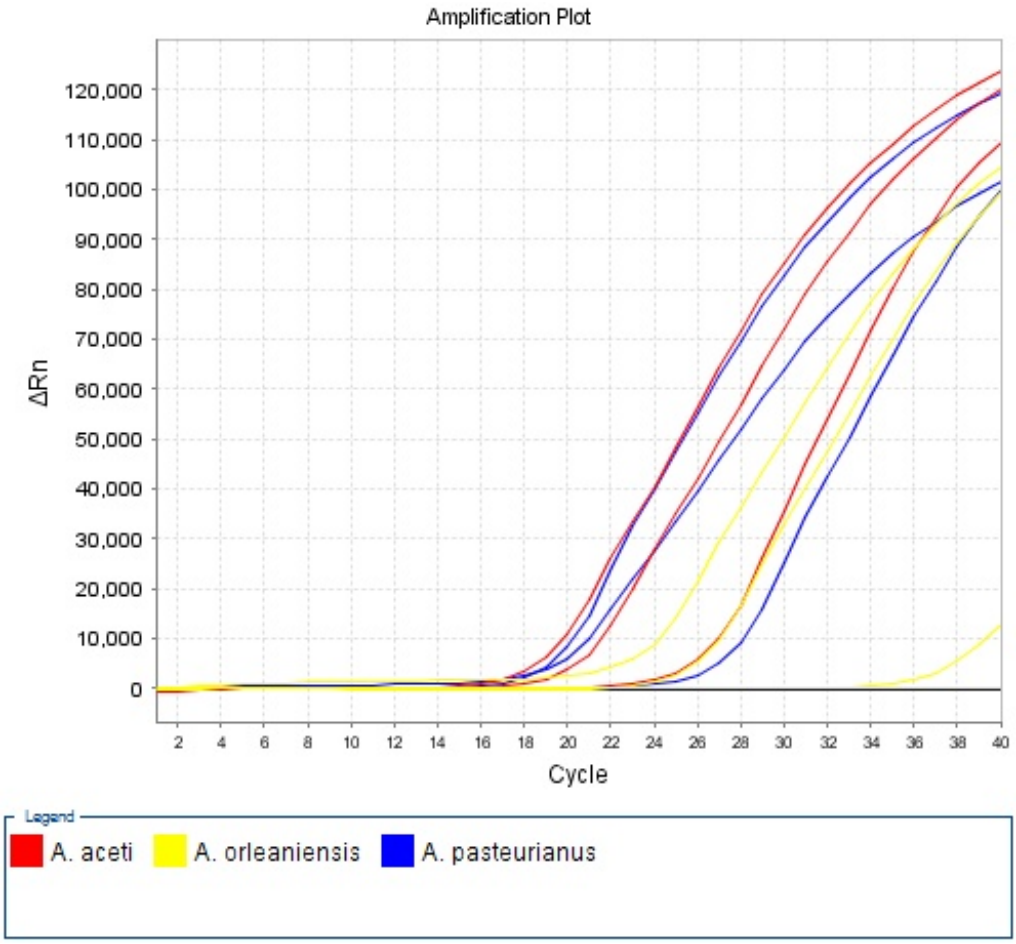

Figure 1 Evaluation of RTQ PCR in cells of *Acetobaacter*

The presence and sensitivity of Gram-negative bacterial species *Acetobacter aceti, A. pasteurianus* and *A. aleaniensis* was detected using Real-Time PCR. Susceptibility of *A. aceti* varied in different isolates from 10^1 to 10^5 CFU.mL^{-1}, the sensitivity of the species *A. pasteurianus* in different isolates of the wine samples ranged from 10^3 to 10^5 CFU.mL^{-1}. We also monitored in the individual isolates representation of species *Acetobacer orleaniensis*, which captured RTQ PCR sensitivity ranging from 10^2 to 10^5 CFU.mL^{-1}.

The two species of *Acetobacter* most often isolated from wine are *A. aceti* and *A. pasteurianus*. More recently a new *Acetobacte*r species, *A. oeni*, has been described which was isolated from spoiled red wine in the Dão region of Portugal (**Silva *et al.*, 2006**). *Acetobacter tropicalis* has recently been isolated from spontaneously fermenting Austrian

wine followed by acetic fermentation (**Silhavy and Mandl 2006**), whereas, previously it had only been isolated from coconut (**Lisdiyanti *et al.*, 2000**).

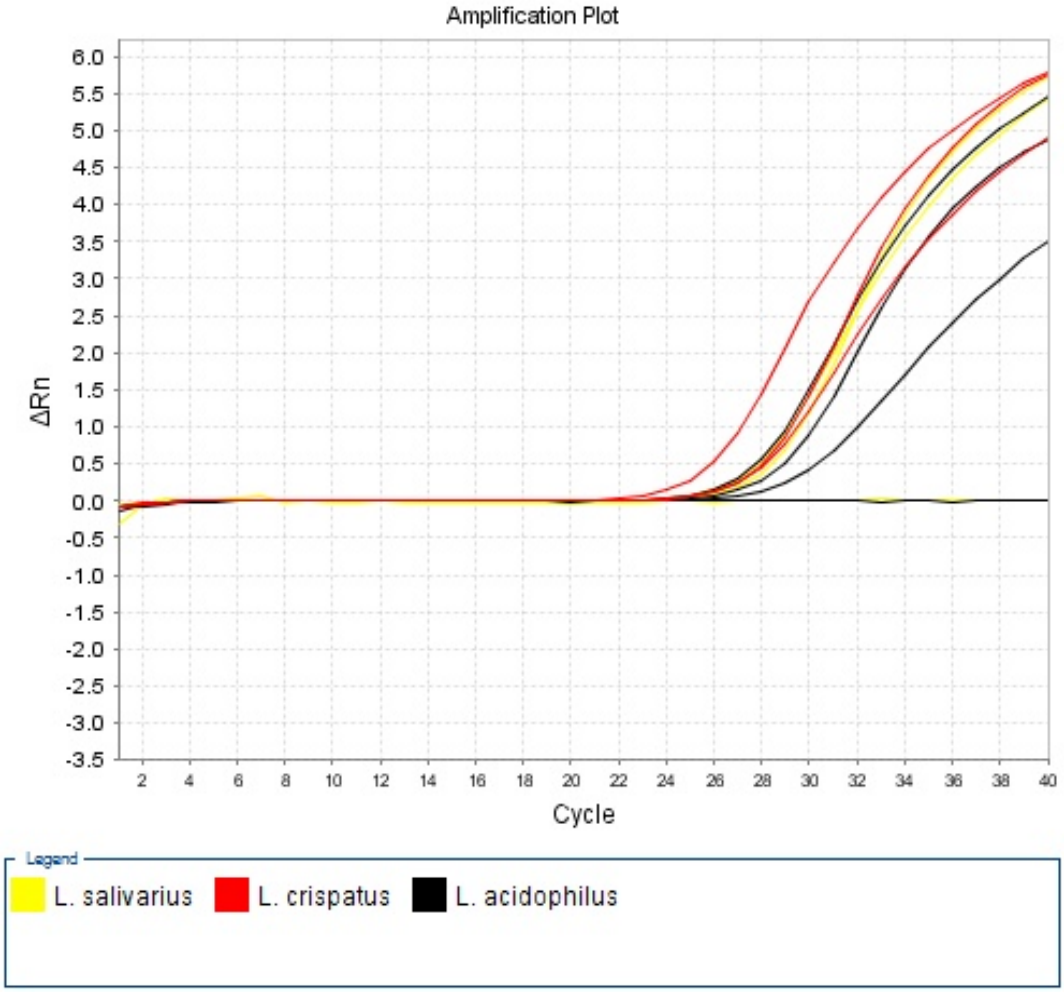

Figure 2 Evaluation of RTQ PCR in cells of *Lactobacilus*

The presence and sensitivity of Gram-positive bacterial species *Lactobacillus salivarius, L. crispatus* and *L. acidophilus* was detected using Real-Time PCR. Susceptibility of *L. salivarius* varied in different isolates from 10^3 to 10^5 CFU.mL^{-1}, the sensitivity of the species *L. crispatus* in different isolates of the wine samples ranged from 10^3 to 10^5 CFU.mL^{-1}. We also monitored in the individual isolates representation of species *Lactobacillus acidophilus*, which captured RTQ PCR sensitivity ranging from 10^3 to 10^5 CFU.mL^{-1}.

Different results with molecular method using for lactobacilli presence of *Lactobacillus brevis, L. casei, L. plantarum, L. hilgardii* and *Leuconostoc mesenteroides* have

been reported in the other studies (**Du Plessis *et al.*, 2004; Canas *et al.*, 2009; Ruiz *et al.*, 2008; 2010**).

CONCLUSION

Although red wines are normally bottled with a low oxygen content, bottles that are stored in a vertical position, leaving a headspace of gas between the surface of the wine in the neck of the bottle and the cork closure will provide the opportunity for oxygen to migrate into the headspace. The oxygen content of this entrapped gas, which is finite, and minimized by the modern bottling equipment used, does not explain the often random nature of the acetic acid and lactic acid bacteria spoilage

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