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IDENTIFICATION OF LACTIC ACID BACTERIA ISOLATED FROM RED WINE SAMPLES BY RT-qPCR

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ABSTRACT

Lactobacillus represents a highly diverse group of Gram-positive, microaerophilic bacteria that microscopically appear as long to short rods or even coccobacilli. The aim of our study were the identification of some species of lactic acid bacteria in red wine during the fermentation process using a classical microbiological and RT-PCR methods. The changes in different groups of microorganisms were monitored in total counts of bacteria, and *Lactobacillus* 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 storage with different conditions. During this period were examined 4 bottles of red wine between Modry Portugal (MP) and Frankovka modra (FM). The total counts of bacteria ranged from 4.21 in the wine MP at 4°C of storage to 5.81 log CFU.mL^{-1} in the wine MP at 25°C of storage, but the total counts of bacteria ranged from 4.85 in the wine FM at 4°C of storage to 5.63 log CFU.mL^{-1} in the wine FM at 25°C of storage. The higher number of lactobacilli cells was found in wine FM at 25°C. The presence and sensitivity of six Gram^{+} bacterial species *Lactobacillus salivarius, L. buchneri, L. plantarum, L. curvatus, L. brevis* and *L. hilgardii* were detected using Real-Time PCR.

Keywords: red wine, fermentation process, *Lactobacillus*, lactic acid bacteria

INTRODUCTION

Lactic acid bacteria (LAB) is a gram-positive, nonsporing, catalase negative in the absence of porphorinoids, aero tolerant, acid tolerant, organotrophic, and a strictly fermentative rod or coccus, producing lactic acid as a major catabolic end product from glucose **(König et al. 2009)**. Species of *Lactobacillus, Oenococcus, Leuconostoc, Pediococcus* and *Weissella* are commonly found on grapes and freshly extracted grape must **(Renouf et al., 2005)**. From the 32 described genera, only 22 species belonging to five genera have been isolated from must and wine **(König et al. 2009)**.

The lactic acid bacteria of grape must and wine belong to the genera *Lactobacillus, Leuconostoc, Oenococcus* and *Pediococcus*. Besides their morphology in coccal or rod-like forms, the homofermentative or heterofermentative character is a deciding factor in their classification. Homofermentative bacteria produce more than 85% lactic acid from glucose. Heterofermentative bacteria produce carbon dioxide, ethanol and acetic acid in addition to lactic acid. Among the cocci, the bacteria from the genus *Pediococcus* are homofermentative and those from the genera *Leuconostoc* and *Oenococcus* are heterofermentative **(Ribéreau-Gayon et al., 2006)**.

LAB can grow in must. Generally they are inhibited at ethanol concentrations above 8%, but *O. oeni* tolerates 14% and *Lactobacillus brevis, Lactobacillus fructivorans* and *Lactobacillus hilgardii* can be found even in fortified wines up to an ethanol concentration of 20%. Slime-producing strains of *Pediococcus damnosus* grow up to 12 % of ethanol. Lactic acid bacteria isolated from wine grow between 15 and 45°C in the laboratory with an optimal growth range between 20 and 37°C. Best growth in must during malolactic fermentation is obtained around 20°C **(König et al. 2009)**.

LAB plays a vital role in reducing wine acidity and also contributing to its aroma and flavour. However, they can also be responsible for many wine spoilage problems that compromise the quality and value of wine. While *O. oeni* contributes positive characteristics to the sensory quality of the wine, species of the genera, *Lactobacillus* and *Pediococcus* can affect the wholesomeness of wine by producing undesirable volatile compounds **(Bartowsky, 2009)**, such as biogenic amines and ethyl carbonate **(Du Toit et al., 2002)**.

Chemical preservatives like sulphur dioxide (SO_2) are used to prevent the growth of spoilage microorganisms during the wine making process **(Bartowsky, 2009)**. SO_2 also acts as a reducing agent and maintains the benefits of antioxidant properties of the polyphenols of wine **(Oliveira et al., 2002; Rojo-Bezares et al., 2007; Bartowsky, 2009)**.

Wine is a microbial diverse environment that harbors microorganisms such as yeasts and bacteria in varying degrees at different oenological stages. The four most relevant oenological genera of lactic acid bacteria (LAB) are *Lactobacillus, Pediococcus, Leuconostoc* and *Oenococcus* **(Bae et al., 2006; Dicks et al., 2009; Ruiz-Larrea, 2010)**.

LAB occurs naturally in the wine ecosystem and plays an essential role in winemaking by reducing its acidity and contributing to the aroma and flavor of the wine. In contrary, they can also be the source of many unwanted wine spoilage problems which reduce the wine quality and value. For instance, unmanaged microbial growth before, during or after wine fermentation can change chemical composition and hence compromise the quality of the end-product. The three stages at which micro-organisms can enter the winemaking process and influence the quality of wine include raw material (grapes), during must fermentation, and post-fermentation. At stage three, wine spoilage can occur in the bottle or during storage in oak barrels **(Du Toit et al., 2002)**.

LAB such as *Oenococcus oeni* contribute positively to the sensory characters of wine, while species like *Lactobacillus spp.* and *Pediococcus spp.* can produce undesirable volatile compounds. Some of the negative effects that result from bacterial wine spoilage include mousy taint, volatile acidity, oily and slimy-texture, overt buttery characters, ropiness, acrolein formation, bitterness, tartaric acid degradation and geranium off-flavour **(Du Toit and Pretorius, 2000)**.

Some LAB can also influence the wholesomeness of wine by producing biogenic amines and ethyl carbonate precursors **(Smit et al., 2008; Lerm et al., 2010; Du Toit et al., 2011)**.

However, topic of food safety and protection of consumers interest is always current and of increasing concern to the general public. Consumer confidence is an essential outcome of a successful food policy and control. EU food safety and its legislation has evolved years, reflecting a blend of scientific, social, political and economic factors **(Horská et al., 2012)**.

The objectives of this study were to investigate the occurrence selected species of lactic acid bacteria in two different Slovakian red wines during fermentation process and to identify the dominant lactic 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 Frankovka modra (FM) and Modry Portugal (MP). The bottles were storage at two different temperatures first at 4°C in refrigerator and second at 25°C at room temperature. The wine FM had content 12% ethylalcohol, 2.1% sugar, 4.6% total acids, 25.4 $g.L^{-1}$ extract and 18.57 $mg.L^{-1}$ SO_2. MP had content 11% ethylalcohol, 1.5% sugar, 5.05% total acids, 24.7 $g.L^{-1}$ extract and 23.85 $mg.L^{-1}$ SO_2.

Determination of CFU counts

For microbiological analysis the wine samples were processed immediately after collection. The total counts of bacteria (TBC), and number of *Lactobacillus* cells (L) 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) in three replications. Homogenized samples of wine were prepared in advance by sequential diluting based on decimal dilution system application. For microorganism cultivation two 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). All lactic acid bacteria (LAB) were cultivated in MRS agar **(De Man et al., 1960)** at 37 °C during 72 hours. Cultivating medium composition corresponded to producer introductions (Biomark™, 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

The LAB reference strains used in this study were as follows: *Lactobacillus brevis* CCM 1815, *Lactobacillus buchneri* CCM 1819^T, *Lactobacillus salivarius* CCM 7274, *Lactobacillus curvatus* CCM 7271, *Lactobacillus hilgardii* CCM 7701 and *Lactobacillus plantarum* CCM 3626. We purchased the pure cultures of bacterial strains from Czech Collection of Microorganisms in Brno.

DNA Extraction

We used two different methods for DNA extraction from bacterial cells.
The first method was very easy: Cells from bacterial cultures were harvested by centrifugation for 5 min. at 10 000 x g and washed with 1 ml of 1 M NaCl twice. The pellet than resuspended in 1 mL water, heated at 105°C and used for PCR analysis.
But for better DNA extraction we used GenElute™ Genomic DNA Miniprep Kit from Sigma Aldrich.
We used procedure for Cultured Cell Preparation:
Lactobacillus species are Gram$^+$ bacteria. The first step was harvested of 72 hours bacterial sample from cell culture. Putt the cell culture to the 2 mL eppendorf tube. Bacterial culture was centrifuged during 5 min / 6.000 to 10.000 g. The supernatants were removed. The pellet was dissolved in 200 µL Resuspension Solution and incubated 2 min. at room temperature. Next were added 20 µL Proteinase K and 200 µL and Lysis Solution C, vortex about 15 second and during 30 min. in 37ºC were incubated. We added 500 µL Column Preparation Solution to each GenElute Miniprep Binding Column (BC), and centrifuged at 12 000 g 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 filtrate were removed and transferred the BC to the new eppendorf tube. Next 500 µL washing buffer was added, then centrifuged at maximum speed unless drying of membrane and then transferred to a new eppendorf tube. The washing buffer used twice. DNA elution: 200 µL of Elution solution directly to the center of the membrane was added, then centrifuged for 1 min. at 6 500 g. The purified DNA was eluted from columns with 150 mL buffer AE and stored at -20°C.

Primers and Real Time PCR

After DNA extraction we were prepared the samples for Real Time PCR. We used Sensifast SYBR Green Hi-ROX kit, specific forward and reverse primer, ultra-pure H_2O and DNA extracted from bacterial samples.
Steps of RTQ PCR: We used 3-step cycling (40 cycles): Polymerase activation 2 min at 95ºC, denaturation 5 sec. at 95ºC, annealing 10 sec. at 60ºC and extension 5 sec at 72ºC. Melt Curve stage 15 sec. at 95ºC and 1 min. at 60ºC. We used Step One™ Thermal cycler from Applied Biosystems®.

Primers are follows

Lactobacillus salivarius	
Lsal-1-F	5'-AATCGCTAAACTCATAACCT-3'
Lsal-2-R	5'-CACTCTCTTTGGCTAATCTT-3'
Lactobacillus brevis	
SCAR-LBR-F	5'-GGAAGATCAAGAATATCGGTG-3'
SCAR-LBR-R	5'-GCGTCTCTAATTCACTGAGC-3'
Lactobacillus plantarum	
SCAR-LPL-F	5'-GAAGATTTGCCCATCGGTG-3'
SCAR-LPL-R	5'-CGTTTGATGGTAGCGTTGC-3 '
Lactobacillus buchneri	
SCAR-LBU-F	5'-CTATCTTTAACCGCATTGCCG-3'
SCAR-LBU-R	5'-GACACGCTTCTCATGATTGTC-3'
Lactobacillus hilgardii	
SCAR-LBH-F	5'-TTCCTTGGTAATGTGCTTGC-3'
SCAR-LBH-R	5'-AATGGCAATCGCAATGGACG-3'
Lactobacillus curvatus	
SCAR-LCU-F	5'-CCAGATCCATCAGAAGATACG-3'
SCAR-LCU-R	5'-GCTAACTTACCACTAACGACC-3'

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)**
The results of microorganisms number in red wine are in table 1. The total number of bacteria ranged from 4.21 to 5.81 log $CFU.mL^{-1}$, the number of lactobacilli ranged from 2.02 to 2.53 log $CFU.mL^{-1}$. The higher number of total count of bacteria was found in Modry Portugal at 25°C of storage. The higher number of lactobacilli was found in Frankovka modra at 25°C of storage. The statistical significant difference was found only between Modry Portugal 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
Modry Portugal at 4°C	**1.**	4.89	2.02
	2.	4.21	2.11
	3.	5.64	2.23
Modry Portugal at 25°C	**1.**	5.81	2.31
	2.	5.58	2.25
	3.	5.62	2.35
Frankovka modra at 4°C	**1.**	5.42	2.36
	2.	4.85	2.47
	3.	5.18	2.44
Frankovka modra at 25°C	**1.**	5.40	2.53
	2.	5.63	2.50
	3.	5.20	2.48

TCB-total count of bacteria, L-*Lactobacilli* cells

Four parameters very distinctly determine the growth rate of lactic acid bacteria in wine: pH, temperature, alcohol content and SO_2 concentration. Other factors are also in play but to a lesser degree and can only be determinant in some conditions. During winemaking, lactic disease is a dreaded bacterial spoilage. By definition, it corresponds with the increase of volatile acidity caused by the heterofermentative fermentation of sugars. Normally, lactic acid bacteria multiply only after the completion of alcoholic fermentation. Lactic disease is also a widespread form of bacterial spoilage in fortified wines. These wines are elaborated by the addition of alcohol to grape must that has been slightly (or not at all) fermented **(Ribéreau-Gayon et al., 2006)**.
Out of about 174 described species/subspecies, sixteen have been found in must and wine **(Ribéreau-Gayon et al. 2006; Fugelsang and Edwards 2007).**

Lactobacillus buchneri, Lb. brevis, Lb. curvatus and Lb. salivarius are commonly found in grape, must and wine. *Lactobacillus plantarum* found in grape must and sewage. *Lactobacillus hilgardii* commonly isolated from wine samples **(König et al., 2009)**.

The presence and sensitivity of six Gram positive bacterial species *Lactobacillus salivarius, L. buchneri, L. plantarum, L. curvatus, L. brevis and L. hilgardii*. It was detected using Real-Time PCR. Susceptibility of *Lactobacillus salivarius, Lb. brevis and Lb. hilgardii* varies in different isolates from 10^3 to 10^5 CFU.mL^{-1}, the sensitivity of the species *L. buchneri* in different isolates of the wine samples ranged from 10^2 to 10^4 CFU.mL^{-1}. *Lactobacillus curvatus* were captured with RTQ PCR sensitivity ranging from 10^2 to 10^3 CFU.mL^{-1}. The last species *Lactobacillus plantarum* had shown very small sensitivity in different isolates, less than 10^1 CFU.mL^{-1}.

The minimal cell count detectable by this identification method using the described conditions was about 10^4-10^5 cells/ml in pure cultures, as well as in mixed populations. According to **(Barata et al. 2012)**, intact grapes contain less than 10^2 CFU.mL^{-1} of Lactic acid bacteria (LAB). So, the initial titre of LAB in must is low, but rises up to 10^4-10^5 CFU.mL^{-1} during the first days of fermentation. During malolactic fermentation the titre can even reach 10^7-10^8 CFU.ml^{-1} **(Lafon-Lafourcade et al., 1983; Lonvaud-Funel et al., 1991)**.

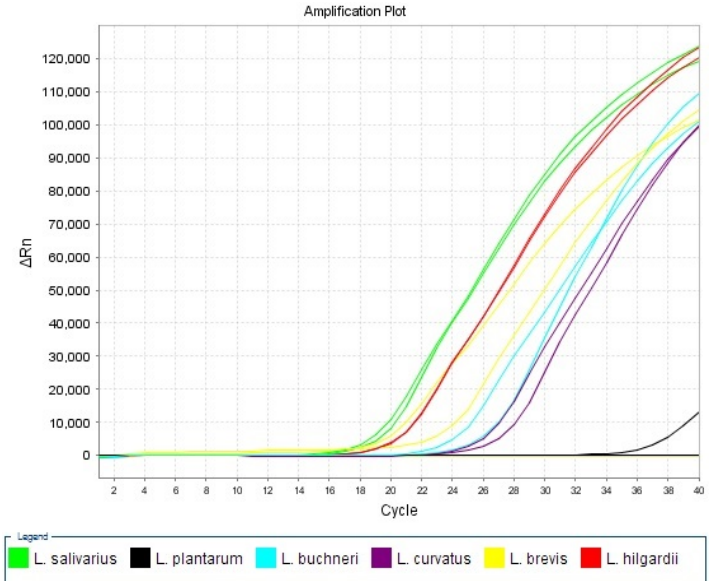

Figure 1 Evaluation of Real-Time PCR in cells of *Lactobacillus*

CONCLUSION

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. LAB are present on grapes, contaminated winery equipment and storage vessels. Some of these bacteria species primarily decompose malic acid and under certain conditions, attack sugar and malic acid. These are often involved in malolactic fermentation and rarely in wine spoilage. When are damaged grapes, low alcohol, acidity and SO_2 concentration, high pH 3.5 and above, temperature more than 25°C, the LAB can cause a wine spoilage. Maintaining a good SO_2 concentration, low pH, and sanitary conditions during processing can prevent the wine spoilage.

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REFERENCES

BAE, S., FLEET, G.H., HEARD, G.M. 2006. Lactic acid bacteria associated with wine grapes from several Australian vineyards. *Journal of Applied Microbiology*, 100, 712-727.

BARATA, A., MALFEITO-FERREIRA, M., LOUREIRO, V. 2012. The microbial ecology of wine grape berries. *International Journal of Food Microbiology*, 153, 243-259.

BARTOWSKY, E.J. 2009. Bacterial spoilage of wine and approaches to minimize it. *Letters in Applied Microbiology,* 48, 149-156

DE MAN, J.C., ROGOSA, M., SHARPE, M.E. 1960. A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology*, 23, 130-135.

DICKS, L.M.T., TODOROV, S.D., ENDO, A. 2009. Microbial interactions. In *Biology of Microorganisms on Grapes, in Must and in Wine*, Eds. H. König, G. Unden and J. Fröhlich. Springer, *Chapter 18*, 335-347.

DU TOIT, M. 2011. *Lactobacillus*: The next generation of malolactic fermentation starter cultures - an Overview. *Food and Bioprocess Technology,* 4, 876-906.

DU TOIT, M., DU TOIT, C., PRETORIUS, I.S. 2002. Biopreservation of wine with antimicrobial peptides. *Bulletin de I'O.I.V. Revue Internationale*, 75, 286-302.

DU TOIT, M., PRETORIUS, I.S., 2000. Microbial spoilage and preservation of wine: Using weapons from nature's own arsenal - A Review. *South African Journal of Enology and Viticulture,* 21, 74-96.

FUGELSANG, K.C., EDWARDS CH.G. 2007. Wine Microbiology: *Practical Applications and Procedures*. 2nd edition. Springer, 2007, 393 p., ISBN 038-7333-41.

HORSKÁ, E. et al. 2012. Food Sciences & Business Studies. Nitra : SPU, 2012, 410 p. ISBN 978-80-552-0815-2.

KÖNIG, H., FRÖHLICH, J., UNDEN, G. 2009. Biology of Microorganisms on Grapes, in Must and in Wine, Springer-Verlag, Berlin Heidelberg, ISBN 978-3-540-85462-3.

LAFON-LAFOURCADE, S., CARRE, E., RIBÉREAU-GAYON, P. 1983. Occurrence of lactic acid bacteria during different stages of the vinification and conservation of wines. *Applied and Environmental Microbiology*, 46, 874-880.

LERM, E. 2010. Malolactic fermentation: The ABC's of MLF. *South African Journal of Enology and Viticulture,* 31, 186-212.

LONVAUD-FUNEL, A., JOYEUX, A., LEDOUX, O. 1991. Specific enumeration of lactic acid bacteria in fermenting grape must and wine by colony hybridization with nonisotopic DNA probes. *Journal of Applied Bacteriology*, 71, 501-508.

OLIVEIRA, C.M., FERREIRA, S., GUEDES, A.C., DE PINHO, P., HOGG, T.A. 2002. Development of a potentiometric method to measure the resistance to oxidation of white wines and the antioxidant power of their constituents. *Journal of Agricultural and Food Chemistry*, 50, 2121–2124.

PETRI, A., PFANNEBECKER, J., FRÖHLICH, J., KÖNIG, H. 2013. Fast identification of wine related lactic acid bacteria by multiplex PCR. *Food Microbiology*, 33, 48-54.

RENOUF, V., CLAISSE, O., LONVAUD-FUNEL, A. 2005. Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. *Australian Journal of Grape and Wine Research,* 11, 316-327.

RIBÉREAU-GAYON, P., DUBOURDIEU, D., DONÉCHE, B., LONVAUD, A. 2006. Handbook of Enology Volume 1, The Microbiology of Wine and Vinifications 2nd Edition. John Wiley & Sons, ISBN 0-470-01034-7.

ROJO-BEZARES, B., SÁENZ, Y., ZARAZAGA, M., TORRES, C., RUIZ-LARREA, F. 2007. Antimicrobial activity of nisin against *Oenococcus oeni* and other wine bacteria. *International Journal of Food Microbiology*, 116, 32-36.

RUIZ-LARREA, F. 2010. Applications of protective cultures and bacteriocins in wine making. In: Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation, Ed. C. Lacroix. Woodhead Publishing Limited, Chapter 17, 432-447.

SMIT, A.Y., DU TOIT, J.W., DU TOIT, M. 2008. Biogenic amines in wine: Understanding the headache. *South African Journal of Enology and Viticulture*, 29, 109-127.