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DEVELOPMENT OF A MOLECULAR METHOD FOR QUANTIFYING *Hanseniospora* POPULATIONS DURING THE WINEMAKING PROCESS

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Abstract

In the wine and in the grape must, there is a complex microbial ecosystem that harbours a great diversity of yeast species, amongst other, non-Saccharomyces yeasts (Issatchenkia orientalis, Metschnikowia pulcherrima, Torulaspora delbrueckii, Candida zemplinina and Hanseniaspora spp.). The paper aimed to develop a molecular method for quantifying Hanseniospora populations during the winemaking process. Specific primers and SYBR Green probe have been used for real-time qPCR method and dilutions from a reference strain of Hanseniospara uvarum, it also hs been used for the calibration curve. After the method was developed, it was used to study different samples of grape must and wine, following a natural or controlled fermentation with commercial yeast strains. This study is necessary, useful and shows the relevance of qPCR for studying non-Saccharomyces yeasts in the complex ecosystem of grape must and wine.

Key words*: qPCR, Hanseniospora, SYBR Green, grape must, wine.*

INTRODUCTION

For the grape must to turn into wine, it is necessary the coexistence and the succession of different yeast species. On the surface of grape grains are present mainly non-*Saccharomyces* (NS) yeasts, being predominant in the early stages of alcoholic fermentation. In the intermediate to the final stages of fermentation, non-*Saccharomyces* yeasts are outgrown by the growth of *Saccharomyces cerevisiae* (Fleet et al., 1993; Fleet, 2003).

To prevent the risk of non-*Saccharomyces* yeasts growing in the beginning of the wine making process, addition of sulphites is a usual industrial method, being considered unattractive in traditional wine making. Nowadays, increased knowledge about yeast diversity has shown that there are several non-*Saccharomyces* yeasts with their own benefits, contributing to the sensory growth and complexity of the wines (Jolly et al, 2014; Carrau et al., 2015; Padilla et al., 2016).

The mechanism involved in the cell death of two *Hanseniospora* species (*Hanseniospora guillermondii* and *Hanseniospora uvarum*) during mixed fermentation, under oenological growth conditions with *Saccharomyces cerevisiae*, was studied by Perez-Nevado et al. (2006). When *S. cerevisiae* reached a cell concentration of close to 10^7 CFU/mL, a reduction in the population of *Hanseniospora* was observed regardless of ethanol concentration. The authors hypothesized that certain toxic compounds produced by *S. cerevisiae* trigger early death of *Hanseniospora* cells.

Mills et al. (2002), using direct molecular methods, detected an active population of *Hanseniospora* strains during fermentation processes, which could not be observed by classical cultivation methods. Hierro et al. (2006a) detected a permanent population of *H. uvarum* and *H. osmophila*, during fermentation processes, not knowing to what extent these species contribute to the total population of yeasts during wine fermentation. The use of molecular methods independent of the classical methods of cultivation on the plate, can lead us as close as possible to the true diversity of yeast populations throughout the fermentative process of wine (Vrajmasu et al., 2018).

In recent studies, microorganisms as *Saccharomyces* and non-*Saccharomyces* yeasts involved in wine making process, have been studied using real-time quantitative PCR

(qPCR) to detect and quantify yeasts strains without the need for plating (Hierro et al., 2007; Zott et al., 2010; Andorra et al., 2011; Portillo et al., 2016).

The aim of this study was to use and develop a molecular method based on DNA (qPCR), to detect and quantify the population levels of different *Saccharomyces* and non-*Saccharomyces* (*Hanseniospora* spp.) yeasts strains during the various stages of alcoholic fermentation process.

MATERIALS AND METHODS

Grape must and microorganisms

In this study, one type of white wine (Feteasca Regală 2018) and two musts (with and without sulphite addition) were tested. Samples were harvested on September 2018 and 50 mg/L of sulphate was added, debourbage of samples was realized next day. The must having 23.2% Brix and 3.94 g/L total acidity, was inoculated with 20g/hL of selected commercial yeasts: Y1, Y2, Y3, Y4, and Y5 (Y1 and Y2 are non-killer *Saccharromyces* strain; Y3, Y4 and Y5 are killer Saccharomyces strain). Grape must samples were coded as M (simple must) and MS (sulphite must). The temperature of fermentation was 16±1.5°C and was conducted during almost 3 weeks. Sampling was done from the first fermentation day till the end of the fermentation; *Hanseniospora* detection was performed in all fermentative phases (lag, exponential and stationary phase).

For the calibration curve was used a strain with a known concentration of *Hanseniospora uvarum* (CSIII2) with a microbial load of 1.4 x 10^{10} cells/mL, from the UASMVB-Faculty of Biotechnology collection.

DNA extraction

Extraction of DNA was performed with Fast ID Genomic DNA Extraction Kit (Genetic ID NA, Fairfield, IA, USA). For each extraction, 1000 µl of Genomic Lyse buffer premixed with 10 µl of Proteinase K solution was mixed with 1000 µl of sample. After a vortex, samples were incubated at 65ºC for 30 minutes, followed by a spin at 10.000 rpm for 5 minutes in a microcentrifuge. Supernatant was passed through the DNA Binding Column. The columns were washed one time with 1000 µl Genomic Wash and three times with 1000 µl of 75% ethanol, after each wash columns were spin for one minute at 10.000 rpm. At the final 100 µl of 1xTE were added and incubated for 10 minutes at 65ºC, followed by a spin at 13.000 rpm for one minute and eluted DNA collected.

The DNA quality was checked using a spectrophotometer (NABI UV/Vis Nano Spectrophotometer, MicroDigital Co. Ltd., Korea).

Real Time PCR method

The DNA samples were treated with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Hampton, NH, USA), in the presence of *Hanseniospora* specific primers Hauf2L (5'-CCCTTTGCCTAAGGTACG-3') and Hauf2R reverse primer (5'-CGCTGTTCTCGCTGTGATG-3') recommended by (Zott et al., 2010) The coupling protocol for one sample is: 12.5 µl SYBR Green qPCR Master Mix, Hauf2L forward primer 0.1 µl (final concentration of 0.3 µM), Hauf2R reverse primer 0.1 µl (final concentration of 0.3 µM), DNA template 5 µl (The DNA concentration in the extracted samples had values between 2.3 and 7.8 ng/µl) and nuclease free water 7.3 µl, total reaction volume 25 µl.

The reaction was performed in a Real-time PCR System (7500 Real-time PCR System, Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) following the program: initial denaturation 10 min at 95ºC; 40 cycles of denaturation 15 s at 95ºC followed by an extension of 60 s at 62ºC. Each sample was amplified in duplicate in every experiment. To generate the standard curve, a 10-fold dilution series of DNA from *Hanseniospora uvarum* strain was subjected to qPCR under the same conditions as described above. All reagents used were molecular biology grade reagents.

RESULTS AND DISCUSSIONS

The purpose of this work was to develop a method for quantifying *Hanseniospora* populations during fermentation in winemaking process. In order to validate the qPCR quantification method, a calibration curve using a *Hanseniospora* strain with a known microbial

loading of 1.4 x 10^{10} cells/mL was required. For the standard curve a very good correlation coefficient was obtained, respectively R^2 = 0.9927 (Figure 1). The limit of detection showed the necessity of the presence of a maximum of Ct equal to 32 for a positive reaction with SYBR-Green. The population level of *Hanseniospora* was obtained by interpolation and expressed in $\log_{10}$ no. cell / mL. The value of efficiency of amplification was 90% for this qPCR reaction, fitting in the validation criteria with an R^2 greater than 0.98 and a slope with a value between -3.1 and -3.6 (Uțoiu et al., 2018).

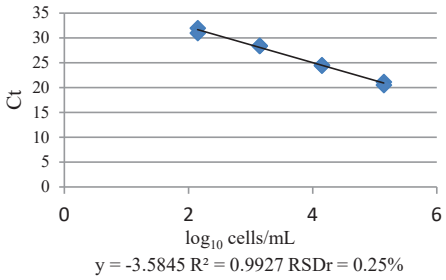

Figure 1. Standard curve obtained from serially diluted *H. uvarum*

Dilutions were performed up to a concentration of 1.4 x 10^2 cells/mL, this value being the limit of detection of the method (Figure 1). The evolution of the *Hanseniospora* population in five types of wine and two musts, were analysed during the alcoholic fermentation, obtaining values for all the analysed samples, the *Hanseniospora* population coming from the grape berries, not being added as a fermentation supplement. Level of *Hanseniospora* population were almost similar for wine samples and significantly higher for wine must samples.

For the Y1 wine samples, the dynamics of the concentration was more constant than in the case of Y2 wine samples, the initial concentration value was 2.91 x 10^4 cells/mL, in the following days of the fermentation period there was a decreasing trend with slight variations, reaching a minimum concentration value on the thirteenth day. 4.15 x 10^3 cells/ mL, so that at the end of the monitoring period, it reaches a maximum recorded value of 3.55 x 10^4 cells/mL, slightly higher than the initial one (Figure 2).

In Y4 wine samples, the initial concentration was 8.4 x 10^4 cells/mL, on the sixth day of fermentation to reach a maximum concentration of 1.18 x 10^5 cells/mL, on the thirteenth day reaching a minimum concentration of 2.84 x 10^4 cells/mL, at the end of the period a concentration of 3.53 x 10^4 cells / mL was recorded (Figure 3). The same trend was registered for Y3 wine samples as for Y5 samples, the initial value recorded was 2.43 x 10^4 cells/mL, on the second day of fermentation the maximum concentration value of 4.22 x 10^4 cells/mL was recorded, the following day recorded a minimum concentration of 8.42 x 10^3 cells/ mL, at the end of the period there was registered a value of 1.94 x 10^4 cells/mL, slightly lower than the initial one (Figure 3). In the Y5 wine samples, an initial concentration value of 5.75 x 10^4 cells/mL was recorded, on the sixth day it reached a maximum value of 8.5 x 10^4 cells/mL, on the fifteenth day of monitoring a minimum value of 7.8 x 10^3 cells/ mL, in the end of the periods a value of 1.9 x 10^4 cells/mL was recorded, a value that is lower than the value of the initial recorded concentration (Figure 3). In Y4 wine samples, the concentration was generally constant, starting from an initial value of 3.62 x 10^4 cells/mL, with slight variations, reaching a minimum value on day 13 of 3.53 x 10^4 cells/ mL, at the end of the period to reach a maximum value of 2.84 x 10^5 cells/mL (Figure 3).

In the case of the must sample, as general remark, the *Hanseniospora* population was higher than in the natural or inoculated wines.

For the simple must samples, an initial concentration value of 4.14 x 10^5 cells/mL was recorded, a slight variation was recorded until the tenth day, when the concentration had a maximum of 4.32 x 10^5 cells/mL and reaching a minimum value on the last day of 2.9 x 10^4 cells/mL (Figure 4). In the case of must samples with sulphites, the highest values of the *Hanseniospora* populations level was recorded in the beginning of the fermentation (6.42 x 10^5 cells/mL): the minimum population level was recorded as 4.36 x 10^5 cells/mL on the eleventh day of fermentation; an increased level of 1.32 x 10^6 cells/mL was recorded on the sixth day. The maximum value of the population level for the sulphite must samples was 2.35 x 10^6 cells/ mL, decreasing slightly on the last day of monitoring at 1.77 x 10^6 cells/mL (Figure 4).

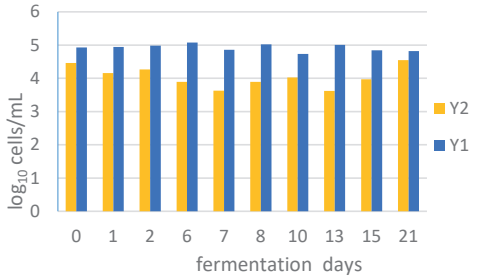

Figure 2. Evolution of *Hanseniospora* population during fermentation with Y1and Y2 non-killer *Saccharomyces* strain

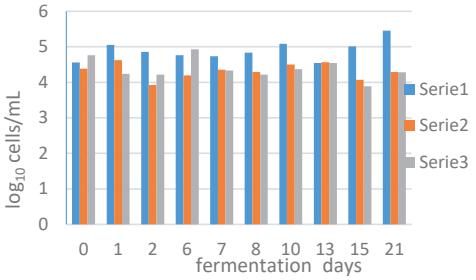

Figure 3. Evolution of *Hanseniospora* population during fermentation with Y3, Y4 and Y5 killer *Saccharomyces* strain

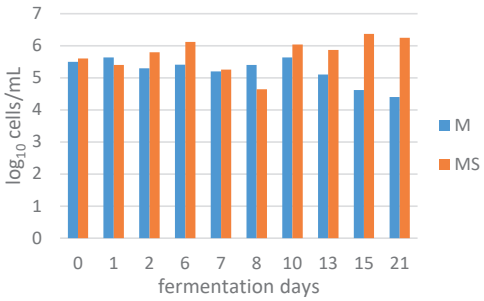

Figure 4. *Hanseniospora* population evolution during fermentation in grape must (M) and grape must with sulphites (MS)

A general image of cardinal *Hanseniospora* level during wine fermentation (beginning of fermentation, end of fermentation and maximum level are presented in Table 1).

In all analysed wine samples, similar values were obtained, with an increasing trend for Y4 wine samples and a decreasing trend for Y3, Y5. Wine samples, Y4 and Y2, registered the minimum and maximum value on the same days (minimum on the thirteenth day and maximum on the last day of monitoring), Y3 recorded the maximum concentration on the second day and Y5 on the sixth day. A difference can be observed between the two types of wine, the concentration of *Hanseniospora* population is slightly higher in the case of killer factor samples than in the case of non-killer ones. Grape must samples had a higher initial concentration than wine, and must samples with sulphites recorded the highest concentrations up to 2.35 x 10^6 cells/mL, with similar results being obtained by Hierro et al. (2007) and Lopez et al. (2015).

Table 1. *Hanseniospora* levels on different wines fermentation stages (cells/mL)

Sample	Beginning of fermentation	End of fermentation	Maximum level
M	$4.1x10^5$	$2.9x10^4$	$4.3x10^5$
MS	$6.42x10^5$	$1.8x10^6$	$2.3x10^6$
Y1	$8.4x10^4$	$6.6x10^4$	$1.2x10^5$
Y2	$2.9x10^4$	$3.5x10^4$	$3.5x10^4$
Y3	$2.4x10^4$	$1.9x10^4$	$4.2x10^4$
Y4	$3.6x10^4$	$2.8x10^5$	$2.8x10^5$
Y5	$5.7 x10^4$	$1.9x10^4$	$8.5x10^4$

Rapid and sensitive methods are needed for yeast detection and enumeration to allow winemakers to control and avoid damaging wines. QuantitativePCR is a fast and accurate technique for quantifying microorganisms associated with food. This technique was used to detect and enumerate the total number of yeasts in wine samples (Martorell et al., 2005; Hierro et al., 2006a; Hierro et al., 2006b; Andorra et al., 2012). In all analysed wine samples, almost similar values were obtained, grape must samples had a higher initial concentration than wine. Must samples with sulphites recorded the highest concentrations up to 2.35 x 10^6 cells/mL, it was assumed that non-*Saccharomyces* yeasts were only present at the beginning of fermentation and were eliminated by the main fermentation yeast *Saccharomyces cerevisiae* (Zott et al., 2010). This method has been applied so far for the enumeration of yeasts from wine by Hierro et al., 2007; Andorra et al., 2012; Rizzotti et al., 2015 and our results are similar to those found by Zott et al., 2010 and Hierro et al., 2007.

CONCLUSIONS

In our effort to set-up a non-culture method to quantify *Hanseniospora* population during wine fermentation, we took a molecular approach by the use of Real Time PCR. For the standard curve a very good correlation coefficient was obtained, respectively $R^2 = 0.9927$ and a value of RSDr = 0.25%. The limit of detection showed the necessity of the presence of a maximum of Ct equal to 32 for a positive reaction with SYBR-Green. The value of efficiency of amplification was 90% for this qPCR reaction, fitting in the validation criteria with an R^2 greater than 0.98 and a slope with a value between -3.1 and -3.6.

In all the analyzed wine samples, almost similar values were obtained for *Hanseniospora* population; grape must samples coefficient was had a higher initial concentration than the wines, and must samples with sulphites recorded the highest concentrations. Our results are in line with other reports and support the idea that qPCR is a fast, direct (non-culture), sensitive and reliable technique for quantifying different yeast species.

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