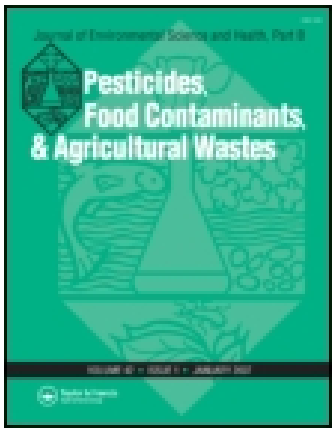

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Determination of wine microbiota using classical method, polymerase chain method and Step One Real-Time PCR during fermentation process

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The aim of our study was the identification of grape, must and 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, lactobacilli and yeasts. Microbiological parameters were observed during the current collection and processing of grapes in 2009. Samples were taken during the fermentation process in wine enterprises and a private vineyard. During this period 30 samples of wine among Müller Thurgau, Cabernet Sauvignon, Chardonnay, Tramin and Red Bio-wine were examined. Samples were collected from stages of grape-must unfiltered, grape-must filtered, the beginning of fermentation, fermentation, late fermentation and young wine. The highest total counts of bacteria ranged from 0.00 to 176 ± 15 CFU.mL^{-1} in the wine of Müller Thurgau, the highest number of yeast ranged from 0.00 to 150 ± 9 CFU.mL^{-1} in the wine of Müller Thurgau and the number of *Lactobacillus* spp. ranged from 0.00 to 92 ± 5 CFU.mL^{-1} in the sample of Cabernet Sauvignon wine. The presence and sensitivity of Gram-positive and Gram-negative bacterial species *Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus crispatus* and *Lactobacillus salivarius* were detected using Real-Time PCR (RTQ PCR). Susceptibility of *Enterococcus faecium* varied in different isolates from 1 to 10^6 CFU.mL^{-1}, the sensitivity of the species *Lactobacillus acidophilus* in different isolates of the wine samples ranged from 1 to 10^5 CFU.mL^{-1}. We also monitored representation of species *Lactobacillus crispatus*, which were captured by RTQ PCR sensitivity and ranged from 1 to 10^5 CFU.mL^{-1}. Identification of the species *Lactobacillus salivarius* in each of isolates by RTQ PCR method showed the presence of these bacteria in the range of 1 to 10^4 CFU.mL^{-1}.

Keywords: Wine, bacteria, yeasts, plate diluting method, PCR method, Real-Time PCR.

Introduction

The microorganisms present in wine-making processes are mainly yeasts, lactic acid bacteria and acetic acid bacteria, because of the extreme conditions in grape must such as the low pH (between 3–4) or the high sugar concentration. *Non-Saccharomyces* yeasts present in the first stages of alcoholic fermentation and *Saccharomyces* species (mainly *Saccharomyces cerevisiae*) are responsible for converting the sugars in grape must into ethanol and CO_2.[1] Lactic acid bacteria decrease the acidity of the wine and convert malic acid into lactic acid and CO_2. This is a one-step reaction known as malolactic fermentation, which usually takes place once the alcoholic fermentation is over.[2]

Acetic acid bacteria (AAB) play a negative role in the wine-making process because they change the organoleptic characteristics of the wine and, in some cases, can also lead to stuck and sluggish fermentations. AAB modify wine, mainly because they produce acetic acid, acetaldehyde and ethyl acetate. They are also involved in other industrial processes of considerable interest for biotechnology such as the production of cellulose, sorbose and vinegar.[3]

Classical microbiological taxonomy has traditionally used morphological and physiological differences among the species to discriminate between them. The tests could only discriminate at the species level, although the physiological methods would not be able to distinguish the currently described species. At the genus level, several characteristics can contribute to the differentiation.[4]

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The main objective of microbial classification is to identify an isolated microorganism up to species level. However, discriminating or typing the different strains or genotypes of a species is gaining increasing importance from an industrial point of view. Therefore, it is important to be able to determine how well each technique can discriminate among strains and to know how many species or strains are involved in the various processes.

Real-Time PCR is a technique that identifies and enumerates bacterial species without having the culture.[5] Authors Campbell and Wright[6] used it to detect viable but non-culturable (VBNC) species of *Vibrio vulnificus*, and also to detect other bacterial species such as the fecal bacteria[7] and *Bacillus cereus*.[8] It is a fast and reliable method for identification and enumeration. Real-Time PCR determines the initial template concentration by continuously measuring the product throughout the reaction and the initial number of cells can also be accurately estimated by comparing it to a standard curve.[9]

The first objective of this study was the identification of grape, must and wine microbiota as total counts of bacteria, number of lactobacilli and yeasts during the fermentation process using a classical microbiological method. The second objective of this study was molecular identification of grape, must and wine microbiota as Gram-negative and Gram-positive bacteria using a classical PCR method and *Lactobacillus* species and *Enterococcus* species using a real-time PCR.

Materials and methods

Microbiological parameters were observed during the current collection and processing of grapes in the year 2009. Samples were taken during the fermentation process in wine enterprises and a private vineyard. During this period were examined 30 samples of wine among five varieties of Müller Thurgau (1 sample), Cabernet Sauvignon (1 sample), Chardonnay (1 sample), Tramin (1 sample) and Red Bio-wine (1 sample). Samples were collected at grape-must unfiltered (5 samples), grape-must filtered (5 samples), the beginning of fermentation (5 samples), fermentation (5 samples), late fermentation (5 samples) and young wine (5 samples).

Determination of CFU counts

For microbiological analysis the wine samples were processed immediately after collection. The total counts of bacteria (TBC), number of yeasts (Y) and number of *Lactobacillus* (L) were assessed. The 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, Y, L) in three replications. Homogenized samples of wine were prepared in advance by sequential diluting based on a decimal dilution system application. For microorganism cultivation three types of cultivating mediums were used, to segregate individual microorganism groups. Glucose Tryptone Yeast agar was used for CFU segregation of TBC (incubation 72 h at 30°C, aerobic cultivation method). Yeast extract agar was used for CFU segregation of Y (incubation 5–7 days at 25°C, aerobic cultivation method) and Lactobacillus MRS agar was used for CFU segregation of lactobacilli (incubation 72 h at 37°C, anaerobic cultivation method). 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 a shaking machine (30 minutes). A prepared basic substance was diluted to reduce the content of microorganisms below 300 CFU level.

DNA analysis

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

G^- bacteria: 1.5 mL of 24 hours bacterial culture was centrifuged 2 min/12.000 to 16.000 g. The supernatant was removed, the pellets were resuspensed in 180 μL lysis solution T, 20 μL proteinase K were added, and incubated for 30 min/55°C, then 200 μL of lysis solution C was added, about 15 s. vortex mixed and incubated at 55°C for 10 min. Next, 500 μL Column Preparation Solution was added to each Gen Miniprep Binding Column, about 12000 g was centrifuged for 1 min., then 200 μL of ethanol (95–100%) was added to lysate and vortex mixed 5–10 s. and about 6500 g was centrifuged for 1 min. The eluate was removed, 500 μL washing buffer was added, then the mixture was centrifuged at maximum speed without drying of the membrane and then transferred to a new Eppendorf tube, to which 200 μL of elution solution was added directly to the centre of the membrane, then the mixture was again 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 pellets were resuspensed in 200 μL lysis solution and incubated for 30 min/37°C, then 20 μL proteinase K was added and incubated for 30 min/55°C, then 200 μL of lysis solution C was added, about 15 s

vortex mixed and incubated at 55°C for 10 min. Next 500 μL of Column Preparation Solution was added to each Gen Miniprep Binding Column and about 12000 g was centrifuged for 1 min, then 200 μL of ethanol (95–100%) was added to the lysate and vortex mixed 5–10 s. then centrifuged about 6500 g for 1 min. The eluates were removed, 500 μL washing buffer was added, then centrifuged at maximum speed without drying of membrane and then transferred to a new Eppendorf tube. An amount measuring 200 μL of elution solution was directly added to the center of the membrane was added, then centrifuged for 1 min. at 6500 g.

Measuring the concentration of DNA-UV-spectrophoto metric quantification of DNA. 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 μg.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} are between 1.8 and 2.0.

Spectrophotometric measurements were done using UV 1101 photometer (Biotech, UK).

Enterococcus faecium. Primer names: FL1, FL2 (550bp). Steps of RTQ PCR: incubation 95°C, 10 min, denaturation 94°C, 10 s., annealing54°C, 10 s., extension 72°C, 30 s., from 2nd to 4th step 35 cycles were repeated, the last step of the operation was temperature 72°C during 10 min.

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

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

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

DNA electrophoresis

Agarose electrophoresis was used to separate the isolated DNA. Agarose (4 g) was mixed with electrophoresis buffer was 1 x TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) and intercalation agent was added. The solution was boiled to a homogeneous state. It was cooled (50°C) and was poured into gel form, later left about 30 minutes at room temperature to harden. After bonding, an electrophoresis comb it was removed and with micropipette was brought into DNA sample and mixed with fluorescent dye (GoldView^{TM}). After spreading of the samples the gel was given into electrophoresis, and sealed by water buffer (TBE).Results from electrophoresis of the separated fractions with UV transilluminator (UniEquip, Mertinsreid) were visualized.

Table 1. Components of PCR reaction.

Components	*Quantity [μL]*
Master mix	15.00
Primer F	0.30
Primer R	0.30
Sample	3.00
PCR water	11.40

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 was used. The 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. Components of PCR reactions are showed in the Table 1.

Statistical analysis of data

The results of microbiological analyses were statistically evaluated by means of Kruskall-Wallis test and Wilcoxon test. Unistat® 5.5 software (Unistat, London, UK) was used for statistical evaluation.

Results and discussion

In many cases, microbial spoilage is not easily defined, particularly in fermented foods and beverages, where the metabolites produced contribute to the flavor, aroma, and taste of the final products. In fact, for cultural or ethnic reasons, there is little difference between what is perceived as spoilage or beneficial activity. The wine production environment may be divided in two fundamental parts: the vineyard, which is a natural ecosystem influenced by cultural practices, and the winery, which is the environment associated with grape fermentation, wine storage and aging, and bottling. A deep knowledge of these two ecosystems—the

Table 2. Yeast counts (YC), lactobacilli counts (LC) and total counts of bacteria (TCB), (CFU mL^{-1}; n = 6; median ± SD) in must and/or wine during winemaking of Müller Thurgau (MT), Cabernet Sauvignon (CS), Chardonay (Ch), Tramine (Tr) and Bio-wine (Bi).

		Phase of winemaking					
Wine	*Group of microorganisms*	*Unfiltered grape-must*	*Filtered grape-must*	*Beginning of fermentation*	*Fermentation*	*Late fermentation*	*Young wine*
MT	YC	10 ± 1^{a}	9 ± 2^{a}	87 ± 8^{b}	150 ± 9^{c}	ND	ND
	LC	87 ± 10^{a}	30 ± 3^{b}	ND	79 ± 5^{a}	ND	ND
	TCB	35 ± 3^{a}	ND**	ND	176 ± 15^{b}	ND	13 ± 1^{c}
CS	YC	10 ± 1^{a}	8 ± 1^{a}	17 ± 1^{b}	88 ± 4^{c}	ND	ND
	LC	69 ± 3^{a}	15 ± 1^{b}	7 ± 2^{c}	92 ± 5^{d}	ND	ND
	TCB	38 ± 5^{a}	19 ± 3^{b}	15 ± 3^{b}	92 ± 5^{c}	ND	ND
Ch	YC	25 ± 4^{a}	$21 \pm 1^{a,b}$	ND	19 ± 2^{b}	ND	ND
	LC	9 ± 3^{a}	ND	ND	17 ± 1^{b}	ND	ND
	TCB	21 ± 2^{a}	17 ± 2^{a}	ND	18 ± 3^{a}	8 ± 3^{b}	ND
Tr	YC	21 ± 2^{a}	12 ± 2^{b}	ND	40 ± 3^{c}	ND	ND
	LC	ND	ND	6 ± 2^{a}	21 ± 3^{b}	ND	ND
	TCB	12 ± 2^{a}	12 ± 1^{a}	ND	14 ± 1^{a}	ND	ND
Bi	YC	70 ± 1^{a}	48 ± 1^{b}	10 ± 1^{c}	60 ± 4^{d}	ND	ND
	LC	71 ± 2^{a}	17 ± 2^{b}	9 ± 2^{c}	40 ± 2^{d}	ND	ND
	TCB	59 ± 7^{a}	51 ± 3^{a}	19 ± 1^{b}	80 ± 6^{c}	ND	ND

*Medians within a line followed by different superscript letters differ ($P < 0.05$).
**ND – not detected.

vineyard and the winery—is essential to establish the origin of wine spoilage yeasts, their routes of contamination, critical points of yeast infection, and their control.[10]

From microbiological parameters in wine samples total counts of bacteria, number of yeasts and lactobacilli were monitored. We also monitored using polymerase chain reaction (PCR) and real time PCR (RTQ PCR) qualitative representation of individual species of microorganisms from the grape, must and wine samples during fermentation.

Table 2 shows the number of yeast, lactobacilli and total counts of bacteria in Müller Thurgau, Cabernet Sauvignon, Chardonnay, Tramin and Red Bio-wine samples which were collected from grape-must unfiltered, grape-must filtered, the beginning of fermentation, fermentation, late fermentation and young wine.

Representation of yeast (Table 2) in different phases of fermentation in Müller Thurgau samples ranged from 0.00 to 150 ± 9 CFU.mL^{-1}. The highest number of yeasts was detected in the phase of fermentation. Presence of yeast in a sample of Cabernet Sauvignon (Table 2) ranged from 0.00 to 88 ± 4 CFU.mL^{-1}. The values of Chardonnay samples (Table 2) ranged from 0.00 to 25 ± 4 CFU.mL^{-1}. Total representation of yeast (Table 2) in different fermentation phases of Tramin samples ranged from 0.00 to 40 ± 3 CFU.mL^{-1}. The values of red bio wine (Table 2) ranged from 0.00 to 70 ± 1 CFU.mL^{-1}. Statistically significant differences ($P < 0.05$) of yeasts in Müller Thurgau were found among unfiltered grape must, beginning of fermentation and the phase of fermentation. Statistically significant differences ($P < 0.05$) of yeasts in Cabernet Sauvignon were found among unfiltered grape must, beginning of fermentation and the phase of fermentation. Statistically significant differences ($P < 0.05$) of yeasts in Chardonnay were found between unfiltered grape must and the phase of fermentation. Statistically significant differences ($P < 0.05$) of yeasts in Tramin were found between unfiltered grape must and the phase of fermentation. Statistically significant differences ($P < 0.05$) of yeasts in Bio-wine were found among unfiltered grape must, filtered grape must, beginning of fermentation and the phase of fermentation.

In general terms, the available information about the presence of microbial communities in vineyards and on grape surfaces may be summarized as follows. Matured grapes harbor microbial populations at levels of 10^3–10^5 CFU.g^{-1}, consisting mostly of yeasts and various species of lactic and acetic bacteria[11] and filamentous moulds; the sources of yeasts and yeast-like microorganisms include all the wine parts, as well as the soil, air, other plants, and animal vectors in the vineyard; insects are the principal vectors for the transportation of yeasts;[12] trust colonization on grapes is influenced by the degree of ripeness of the bunch[13]; the occurrence and growth of microorganisms on the skin of the grapes is affected by the rainfall, temperature, grape variety, and application of agrochemicals[14,15]; yeasts are mainly localized in areas of grape surface where some juice might escape and are embedded in a fruit secrete. The outer surface of the berries is covered by a waxy layer, which affects the adherence of microbial cells and their ability to colonize the surface; oxidative basidiomycetous

yeasts, without any enological interest are mostly prevalent in the vineyard environment (soil, bark, leaves, grapes)[15]; apiculate yeasts and oxidative yeasts are predominant on ripe sound grapes.[15]

The association between the winery and this species is so close that these authors called it "the first domesticated microorganism" and claim that it is a result of yeast species evolution in this environment.[10]

Representation of *Lactobacillus* spp. (Table 2) in Müller Thurgau samples during various phases of fermentation ranged from 0.00 to 87 ± 10 CFU.mL^{-1}. The values of Cabernet Sauvignon (Table 2) ranged from 0.00 to 92 ± 5 CFU.mL^{-1}. Total representation of the lactobacilli number in Chardonnay samples (Table 2) ranged from 0.00 to 17 ± 1 CFU.mL^{-1}. Representation of *Lactobacillus* spp. (Table 2) in Tramin various phases of fermentation ranged from 0.00 to 21 ± 3 CFU.mL^{-1}. The lactobacilli values in red Bio-wine (Table 2) ranged from 0.00 to 71 ± 2 CFU.mL^{-1}. Statistically significant differences ($P < 0.05$) of lactobacilli in Müller Thurgau were found between unfiltered grape must and filtered grape must. Statistically significant differences ($P < 0.05$) of lactobacilli in Cabernet Sauvignon were found among unfiltered grape must, filtered grape must, beginning of fermentation and the phase of fermentation. Statistically significant differences ($P < 0.05$) of lactobacilli in Chardonnay were found between unfiltered grape must and the phase of fermentation. Statistically significant differences ($P < 0.05$) of lactobacilli in Tramin were found between beginning of fermentation and the phase of fermentation. Statistically significant differences ($P < 0.05$) of lactobacilli in Bio-wine were found among unfiltered grape must, filtered grape must, beginning of fermentation and the phase of fermentation.

Another group of microorganisms was total counts of bacteria. The data indicate that the numbers of total counts of bacteria in the Müller Thurgau (Table 2) samples ranged from 0.00 to 176 ± 15 CFU.mL^{-1}. The total counts of bacteria in Cabernet Sauvignon sample (Table 2) ranged from 0.00 to 92 ± 5 CFU.mL^{-1}.The values of Chardonnay (Table 2) ranged from 0.00 to 21 ± 2 CFU.mL^{-1}. The values of Tramin ranged from 0.00 to 14 ± 1 CFU.mL^{-1}. The data indicate that the total counts of bacteria in the Bio-wine samples ranged from 0.00 to 80 ± 6 CFU.mL^{-1}. Statistically significant differences ($P < 0.05$) of total count of bacteria in Müller Thurgau were found among unfiltered grape must, filtered grape must, beginning of fermentation and young wine. Statistically significant differences ($P < 0.05$) of total count of bacteria in Cabernet Sauvignon were found among unfiltered grape must, filtered grape must, beginning of fermentation and the phase of fermentation. Statistically significant differences ($P < 0.05$) of total count of bacteria in Chardonnay were found between unfiltered grape must and the phase of late fermentation. Statistically significant differences of total count of bacteria in Tramin were not found. Statistically significant differences ($P < 0.05$) of total count of bacteria in Bio-wine were found among unfiltered grape must, filtered grape must, beginning of fermentation and the phase of fermentation.

Table 3. Statistical evaluation of concentration of DNA Gram-positive and Gram-negative bacteria in wine samples.

Bacteria group	*n*	$\bar{x}$	x_{min}	x_{max}	*CV%*	*SD*
Gram negative	5	0.023	0.019	0.031	21.64	0.005
Gram positive	5	0.019	0.016	0.023	14.41	0.002

n - sample number, $\bar{x}$ - mean, x_{min}- minimum, x_{max}- maximum, V - coefficient of variation, SD - standard deviation.

DNA of individual wine samples was isolated from pure cultures of bacteria from different samples of wine during the fermentation process. The absorbance measured at a wavelength 260 nm was found in Gram-negative bacterial DNA concentration (Table 3) and ranged from 0.019 to 0.031 $\mu g.\mu L^{-1}$. The average concentration of DNA G$^-$bacteria was 0.0232 $\mu g.\mu L^{-1}$. The concentration of DNA Gram-positive bacteria ranged from 0.016 to 0.023 $\mu g.\mu L^{-1}$. The average concentration of DNA G$^+$ bacteria was at 0.0190 $\mu g.\mu L^{-1}$. Using the specific primers we demonstrated the presence of Gram-positive and Gram-negative bacteria.

Winemaking involves a mixed culture of numerous microorganisms including fungal, yeast, and bacterial species. The principal bacteria present in wine are members of the lactic acid bacteria (LAB). They can transform a large variety of organic compounds present in must or wine, giving final products that affect the organoleptic characteristics of wine. LAB can be detrimental or beneficial depending on the species and the vinification moment at which they develop. A lot of lactobacilli have been isolated as responsible for lactic acid spoilage from sluggish fermentations and sweet fortified wines.[16]

Authors Stratiotis and Dicks[17] described that *L. vermiforme* was among the lactobacilli species that are the most frequently isolated from South African fortified wines. Previously, DNA hybridization studies were performed by Farrow et al.[18] on three strains identified as *L. vermiforme* indicated that they shared a high DNA homology with the type strain of *L. hilgardii*. Based on these results, the species named *L. vermiforme* was rejected. Since the identification of wine heterofermentative lactobacilli is often ambiguous, recently, research has focused on the application of molecular biology methods for microbial identification. LAB species included in the *L. casei* and the *L. plantarum* groups were differentiated based on their recA sequences.[19,20]

Detection of microorganisms by Real-Time PCR

The presence and sensitivity of Gram-positive and Gram-negative bacterial species *Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus crispatus* and *Lactobacillus*

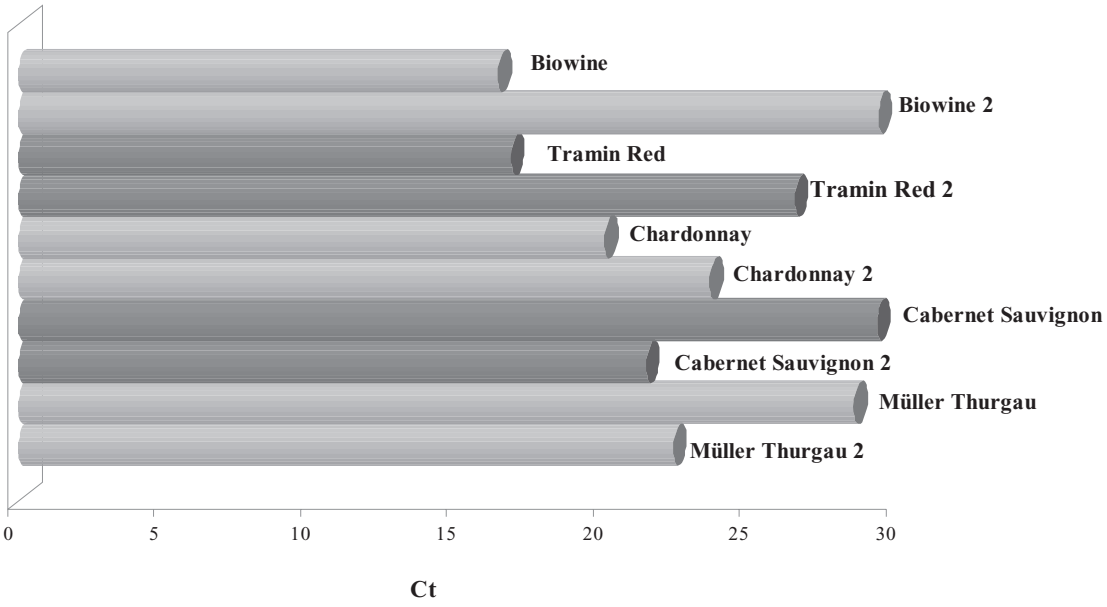

Fig. 1. Evaluation of RTQ PCR in cells of *Enterococcus faecium*.

salivarius was detected using Real-Time PCR. Susceptibility of *Enterococcus faecium* varied in different isolates from 1 to 10^6 CFU.mL^{-1}, the sensitivity of the species *Lactobacillus acidophilus* in different isolates of the wine samples ranged from 1 to 10^5 CFU.mL^{-1}. We also monitored in the individual isolates representation of species *Lactobacillus crispatus*, which captured RTQ PCR sensitivity ranging from 1 to 10^5 CFU.mL^{-1} and identification of the species *Lactobacillus salivarius* in each of isolates by RTQ PCR method, we found that the presence of these bacteria was in the range of 1 to 10^4 CFU.mL^{-1}.

Enterococcus faecium was detected in all tested samples (Fig. 1). The threshold value was 1278.28 by *E. faecium* samples. The (Ct) value of positive samples was on average 23.44 whereby the lowest value of positive samples was found at 16.39 and the highest value was at 29.32. In our samples we could detect strain of *Lactobacillus acidophilus* in seven out of ten samples (Fig. 2). The threshold value was 712.72 by *L. acidophilus* samples. The (Ct) value of positive samples was on average 33.53 whereby the lowest value of positive samples was found at 19.16 and the highest value was at 40.61. In our samples we could detect strain of *Lactobacillus crispatus* in six out of ten samples (Fig. 3). The threshold value was 1.76 by *L. crispatus* samples. The (Ct) value of positive samples was on average 18.35 whereby the highest value was at 24.25 and the lowest value of positive samples was found at 11.16.

Thus, the PCR-based detection of bacteria depends on the efficiency of DNA extraction procedure used to prepare the template DNA. We could detect strain of *Lactobacillus*

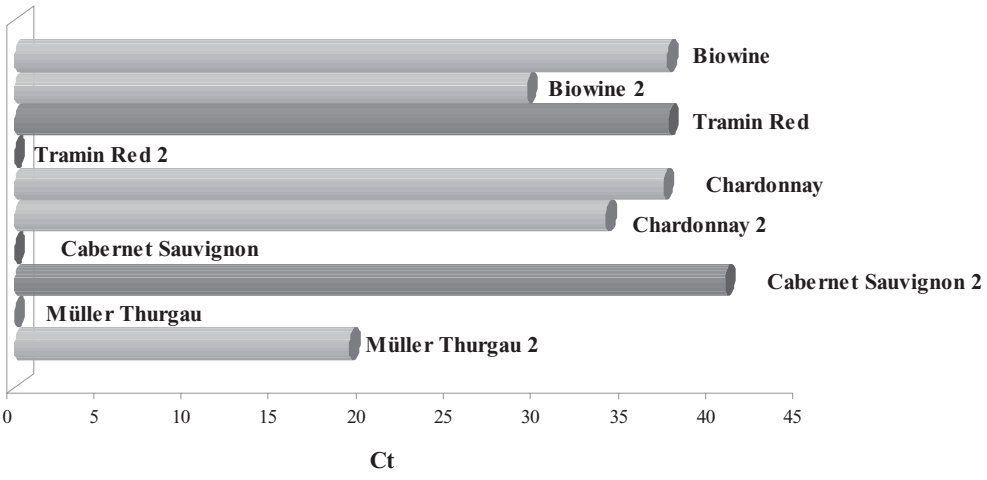

Fig. 2. Evaluation of RTQ PCR in cells of *Lactobacillus acidophilus*.

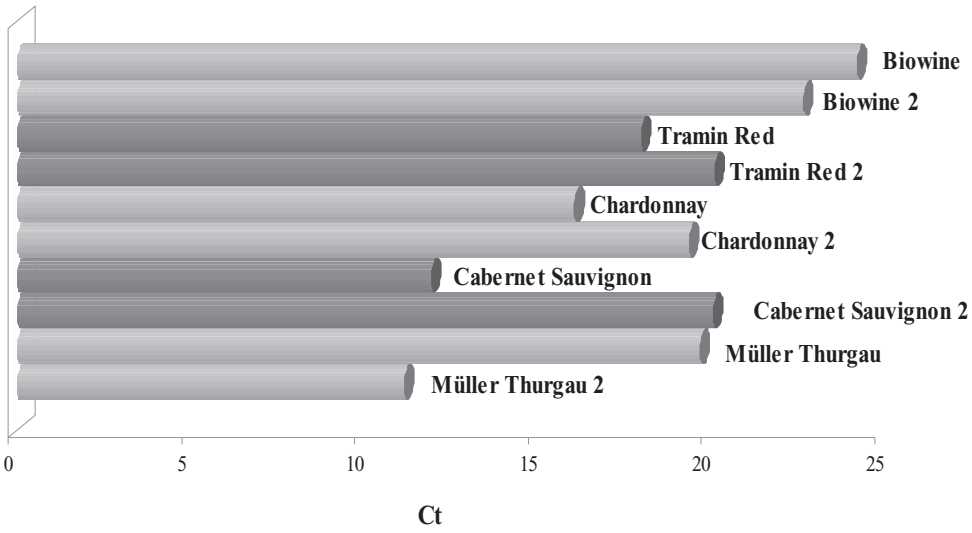

Fig. 3. Evaluation of RTQ PCR in cells of *Lactobacillus crispatus*.

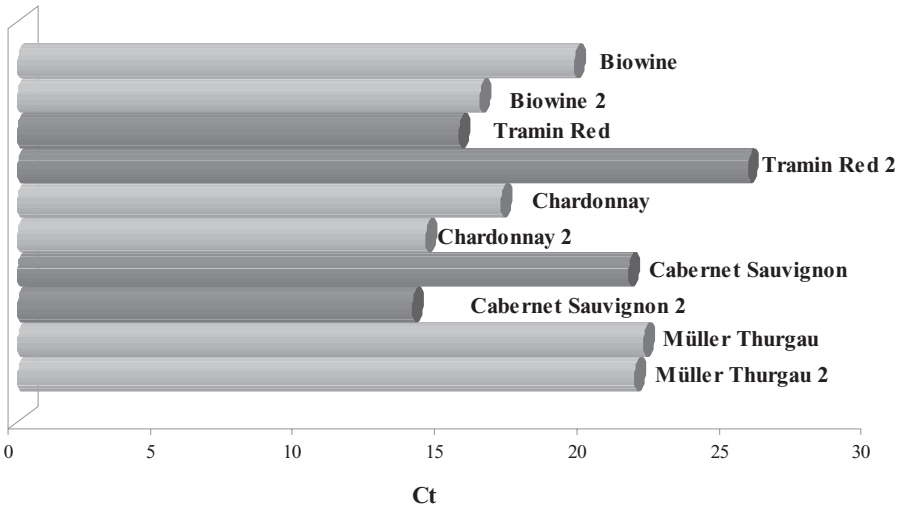

Fig. 4. Evaluation of RTQ PCR in cells of *Lactobacillus salivarius*.

salivarius in all tested samples (Fig. 4). The threshold value was 1175 by *L. salivarius* samples. The (Ct) value of positive samples was on average 18.75 whereby the lowest value of positive samples was found at 13.83 and the highest value was at 25.68.

Different results with molecular method using for lactobacilli presence of *Lactobacillus brevis, L. casei, L. plantarum, L. hilgardii* and *Lc. mesenteroides* have been reported in the other studies.[21–24]

Conclusion

Ecological surveys performed in vineyards and on grape surfaces during ripening are relatively few when compared to those performed on grape musts and on their spontaneous fermentation. Moreover, the majority of them used less optimal sampling, pre-isolation techniques, enrichment methods, isolation of culture media, and incubation times, leading to an insufficient knowledge of grape microbial ecology. The establishment of acceptable levels of microorganisms in the final product is a concern of many food industries. The aim of the wine producer is to comply with levels that are attainable under industrial conditions and ensure product stability during its shelf life. While comparing our results with classical microbiological methods and polymerase chain reaction of comparing our results we found, that traditional methods detected only a quantitative representation of microorganisms, while using methods of molecular biology, we are able for a relatively short time to determine the quantitative but also qualitative presence of microorganisms. For rapid detection of microorganisms in oenological practice, we recommend use of the polymerase chain reaction and in particular Real-Time PCR.

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