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A Survey on Acetic Acid Bacteria Levels and Volatile Acidity on Several Wines of the Republic of Moldova

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Abstract: Acetic acid bacteria (AAB) are ubiquitous wine spoilage microorganisms causing significant economic damage to winemakers. Considering difficulties in their isolation by traditional microbiological methods, it would be advantageous to detect them by molecular methods at all stages of winemaking and thus prevent wine spoilage. In this research, we analyzed wines, musts and grapes of 13 varieties grown in different regions of the Republic of Moldova. The DNA was extracted and analyzed by PCR with home-designed primers to detect *Acetobacter aceti* and *Acetobacter pasteurianus*. Generally, samples with no detectable amounts of AAB in either musts or wine had volatile acidity within the acceptable limits. Only one grape (*Rara Neagra*) had detectable amounts of AAB (*A. pasteurianus)* at all analyzed stages (grape, must, wine), and this sample had the highest amount of volatile acidity (2.11 g/L), exceeding the maximum acceptable limit for red wines of 1,2 g/L. *A. pasteurianus* was more common than *A. aceti* both in musts and wines. Samples positive for AAB but containing low amounts of them in wine (Cq value >35) did not have volatile acidity above the acceptable level. Samples with wine negative but must positive for AAB had volatile acidity close to the acceptable limit. This research shows the perspective of PCR diagnostics for predicting the risks of wine spoilage by AAB.

Keywords: acetic acid bacteria; wine spoilage; primers; real-time PCR; volatile acidity

1. Introduction

Acetic acid bacteria (AAB) are very widespread spoilage microorganisms in winemaking, exert a negative effect on the quality of wines and require the close attention of winemakers at all stages of wine production and storage [1]. These bacteria are obligate aerobes, well adapted to high level of sugars and ethanol [2], have high requirement for the presence of oxygen. When these AAB are present during winemaking, aging or wine storage, they metabolize ethanol to acetaldehyde by alcohol dehydrogenase and then produce acetic acid by acetaldehyde dehydrogenase [3], produce acetoin from lactic acid and ethyl acetate, metabolize glycerol to dihydroxyacetone [4]. Besides, they seem to affect wine quality by influencing must composition and alter the growth of yeast and lactic acid bacteria during fermentation [5].

AAB species typically associated with grapes and must is *Gluconobacter oxydans* which prefers a sugar rich environment [3, 6, 7,] while the ones associated with wine are *Acetobacter aceti* and *Acetobacter pasteurianus* which prefer ethanol as a carbon source [3, 6, 8, 9].

Acetic acid is the main component of the volatile acidity of grape musts and wines. It can be formed as a by-product of alcoholic fermentation or as a product of the metabolism of acetic and lactic acid bacteria, which can metabolize ethanol and residual sugars to increase volatile acidity [10]. The presence of wild yeasts (e.g. *Brettanomyces* and

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its anamorph *Dekkera*, *Pichia anomala, Kloeckera apiculata* and *Candida krusei*) lead to acetification of wine above objectionable levels [4]. Volatile acidity should be measured, at minimum after primary and malolactic fermentation, periodically through wine storage, when a film is found on a specific wine, pre-bottling [11].

The European regulation (CE 1308/2013) has set out limits for sale at 1.20 and 1.08 g/L acetic acid for red wines and white/rose wines, respectively [3], as well as the legislation of the Republic of Moldova. These limits are provided by regulation regarding the organization of the wine market in Republic of Moldova, GD No. 356 from 11-06-2015, p. 38/4.

Several strategies have been applied to prevent wine spoilage by microorganisms during the production. Primary strategies that could be mentioned are compliance with hygiene rules and regulations at wineries, monitoring of nutrients and residual sugars during the fermentation and at the end of it, temperature control, use of sulphur dioxide, the use of purified enzymes for the maceration or clarification of wines, filtering wines with little concentration of sulphur dioxide and a high pH and avoiding the use of old oak barrels for aging the wines.

Detecting and quantifying methods of the harmful microorganisms in winemaking are essential to prevent wine spoilage. These methods can be conventionally divided into two groups: microbiological and molecular methods. The conventional microbiological methods are inexpensive and simple-to-perform, however they are time consuming (1 to 2 weeks), laborious and limited in their ability to detect microorganisms in viable but non-culturable state [12] or microorganisms still difficult to cultivate on laboratory media, which highlights the importance of alternative methods of detection of these bacteria [7]. Also, traditional methods require trained personnel and a final identification is preformed through biochemical, physiological and morphology analysis via a microscopic examination, increasing the overall cost and limiting the test to the lab settings [13].

Recently, direct or indirect molecular-based methods have been applied to overcome the limitations of microbiological methods [14]. Indirect methods include a traditional microbiological step – plating or enrichment, followed by the molecular identification of microorganisms. Direct methods imply detecting and identifying the microorganism directly from the sample at any stages of wine-making (grape, must, wine). Generally, direct methods have two major advances over the indirect methods. First, they can identify non-culturable microbe (those injured, viable but non-culturable, or unable to grow on the chosen media). Second, the direct methods are much faster than indirect, since some microorganisms may require up to two weeks to grow [14]. In winemaking, timely detection of these microrganisms can be crucial to prevent wine spoilage and economical losses, so the development of affordable rapid direct methods suitable for on-site analysis is the priority. Molecular biology methods, such as quantative PCR (qPCR), demonstrate high efficiency in early detection and quantify AAB and can be widely used in the winemaking process [15-17]. Quantitative real-time PCR assay, used in our research, is automated, sensitive, and rapid since it reduces or even eliminates lengthy enrichment and isolation processes [18]. It can also quantify PCR products with greater reproducibility while eliminating the need for post-PCR processing, thus preventing carryover contamination.

2. Materials and Methods

2.1. *Collection of samples*

Grape samples were collected from different regions with Protected Geographical Indication (PGI) – *Codru, Stefan Voda* and *Valul lui Traian* (Figure 1) [19].

Figure 1. Winemaking regions of Republic of Moldova with Protected Geographical Indication (PGI) [19].

Three samples of each of the following grape varietis are used in this study at three stages of winemaking: Rkatiteli, Feteasca Neagra, Augustina, Ametist, Feteasca Regala, Pinot Gris, Alexandrina, Nistreana, Viorica, Cabernet Petit, Rara Neagra, Feteasca Alba, Chardonnay. They belong to four major groups: international varieties (Pinot Gris, Cabernet Petit, Chardonnay), local Georgian varieties grown in Moldova as well (Rkatiteli), local Moldavian-Romanian varieties (Feteasca Neagra, Feteasca Alba, Feteasca Regala, Rara Neagra) and local Moldavian new selection varieties (Augustina, Ametist, Alexandrina, Nistreana, Viorica).

Most varieties were grown in Codru PGI region, except for two varieties grown in Stefan Voda PGI region (Feteasca Neagra-Purcari and Rara Neagra) and two varieties grown in Valul lui Traian PGI region (Chardonnay and Feteasca Regala-Cahul).

The 2021 year samples were collected at three stages of wine-making: stage I – collecting and processing of grapes; stage II – must production, stage III – wine production after clarification and stabilization, before bottling after clarification and stabilization, before bottling.

2.2. *Isolation of the wine DNA*

For DNA isolation from grapes, 150 g of grapes were washed in PBS buffer for 20 minutes, buffer was centrifuged at 5000g for 20 minutes, pellet was resuspended in 0.6mL of extraction buffer, and further extraction was carried out following the same protocol as must and wine samples [20].

Ten ml of each wine or must sample was centrifuged at 5000 g for 30 minutes. The pellet was resuspended in 0.6 mL of the extraction buffer (Tris-HCl 0.2M pH 8.0, NaCl 0.25M, Na2EDTA 0.025M, SDS 5 %w/v) and heated at 65°C for 1 hour. All reagents were molecular biology grade (Sigma-Aldrich). Then 60 mg of PVP powder and with 0.5 volume of ammonium acetate solution (7.5 M) was added to the sample and incubated on ice for 30 min. After 10-minute centrifugation at 10000 g the supernatant was transferred to a fresh tube, mixed with equal volume of chloroform, vortexed and centrifuged again at 10000g. The upper phase was transferred to the new tube, mixed with equal volume of isopropanol and incubated at -20°C for 30 minutes. The samples were centrifuged, the pellet washed twice with 70% ethanol, air dried and dissolved in 50 µL of water; 2 µL of the resulting DNA solution was used per PCR reaction. DNA quality and concentration were checked spectrophotometrically using Genova Nano micro-volume spectrophotometer.

2.3. *Real-time PCR amplification*

Polymerase chain reactions (PCR) was done in real-time PCR Detection Systems CFX96 TouchTM BIORAD. The PCR cycling conditions were as recommended by SYBRGreen manufacturer (Applied Biosystems): 95ºC for two minutes as initial denaturation step followed by alternations of 95ºC for 15 sec and 60ºC for 1 minute for 40 cycles. For melting curve construction, samples were heated to 95ºC for 15 seconds, then incubated at 60ºC for 1 minute (1.6ºC /sec ramp rate), then heated to 95ºC for 15 seconds (0.15ºC/sec ramp rate). The detection of the amplified product was done at SYBR channel.

Previously described primers based on the sequence AB161358.1 (*Acetobacter aceti* genes for 16S rRNA, 16S-23S rRNA ITS and 23S rRNA) were used for *A. aceti* detection (P173–TTTTGAAATGTGACGCGCTTGAATG, P174–TTGCTCCCATGCACAGAAACC); and previously described primers based on the sequence AJ888874.1 (*Acetobacter pasteurianus* partial adhA gene for alcohol dehydrogenase), (P175–CCGGCGGTGATCTTCTGTTC, P176–CCGCTCTGTGCGTCAAACTT) were used for *A. pasteurianus* detection [20].

2.4. *Calculations of relative Cq values*

qPCR cycle threshold (Cq) values represent the number of amplification cycles required for the fluorescent signal to exceed the basal threshold level. Cq values are inversely related to the number of copies of the target gene in a sample, meaning that lower Cq values correlate with higher pathogen loads [21]. However, these values can be difficult to interpret since they have inverse correlation with the pathogen amount. On the other hand, knowing the exact amount of pathogen may not be necessary for the particular experimental purpose, but rather, a comparative study of infection load between samples may be quite informative. To get a visual interpretation of the infection load in different samples, we analyzed the qPCR data by subtracting the Cq value obtained for a given sample from Cq value=40, which is the number of cycles in the PCR reaction, and corresponds to the minimal amount of target gene which can be detected in this assay. Thus, the difference between the actual Cq value and Cq value of 40 indicates how sooner the fluorescent signal exceeds the threshold level in the sample, compared to the theoretical minimal amount corresponding to 40 cycles. The greater the difference is, the more target gene initially contained in the sample, the higher infection load was in the sample.

2.5. *Calculations of relative amount of A. pasteurianus*

For calculation of relative amount of *A. pasteurianus*, the amount corresponding to 40 amplification cycles was taken as a reference point. Since the amount of the DNA doubles at each cycle, one can calculate the fold increase in the DNA amount in different samples compared to the reference point by putting 2 to the power of calculated relative Cq value.

2.6. *Measurement of the volatile acidity in wine*

Volatile acidity was determination by steam distillation/titration, method OIV-MA-AS313-02: R 2015 from Compendium of International Methods of Analysis – OIV [22].

2.7. Statistical Analysis

The experiments in this research were performed in triplicate. One-way analysis of variance (ANOVA) was performed according to Tukey's test at a significance level of $p \leq 0.05$ with Staturphics software, Centurion XVI 16.1.17 (Statgraphics Technologies, Inc., The Plains, VA, USA).

3. Results and Discussion

In this work, we studied the distribution of two *Acetobacter* species (*A. aceti* and *A. pasteurianus*) in wine samples at different stages of wine making (Table 1). The primers p173-174 correspond to *A. aceti* and p175-176 correspond to *A. pasteurianus*.

Table 1. *A. aceti* and *A. pasteurianus* qPCR Cq values and volatile acidity in grapes, musts and wines at different stages of wine production.

Nr.	**Varieties**	**Grapes**		**Must**		**Wine**		**Volatile acidity*, g/L**
		Primers						
		P173-174	**P175-176**	**P173-174**	**P175-176**	**P173-174**	**P175-176**	
1	Rkatiteli	N/A	N/A	N/A	N/A	33,34±2,09	N/A	0,71
2	Feteasca Neagra, Purcari	N/A	N/A	N/A	N/A	N/A	N/A	0,85
3	Augustina	N/A	N/A	N/A	N/A	N/A	37,69±0,84	0,51
4	Ametist	N/A	N/A	N/A	34,46 ±0,41	N/A	N/A	0,58
5	Feteasca Regala, Cricova	N/A	N/A	N/A	32,12 ±0,41	N/A	N/A	0,70
6	Pinot Gris	N/A	N/A	N/A	33,25±0,21	N/A	N/A	0,66
7	Alexandrina	N/A	N/A	N/A	N/A	N/A	34,74±0,80	0,41
8	Nistreana	N/A	N/A	N/A	34,50± 0,57	N/A	31,73±0,79	0,51
9	Feteasca Neagra, Nisporeni	N/A	N/A	N/A	N/A	N/A	N/A	0,85
10	Viorica	N/A	N/A	35,62±1,13	N/A	N/A	31,19±0,51	1,73
11	Cabernet Petit	N/A	N/A	N/A	30,22± 0,48	N/A	N/A	1,23
12	Rara Neagra	N/A	30,08±0,11	N/A	27,02± 0,20	N/A	29,31±0,34	2,11
13	Feteasca Alba	N/A	N/A	N/A	N/A	N/A	36,07±1,05	0,50
14	Feteasca Neagra, Milesti Mici (MM)	N/A	N/A	N/A	30,84± 0,40	N/A	N/A	0,57
15	Feteasca Regala, Orhei	N/A	N/A	36,73±1,24	36,02±0,02	N/A	N/A	0,73
16	Chardonnay	N/A	N/A	N/A	N/A	N/A	N/A	0,80
17	Feteasca Regala, Cahul	N/A	N/A	N/A	N/A	N/A	36,79±1,40	0,85

*Admissible limits of volatile acidity (expressed as acetic acid): for white wines –1,08. g/L, for red wines 1,2 g/L (expressed as acetic acid); $p \leq 0.05$.
N/A – below the detection limit. The mean Cq value and standard deviation were. calculated for three replicates.

As it can be inferred from figure 2, *A. pasteurianus*, infecting 1 grape sample (5.8%), 8 must samples (47%) and 7 wine samples (41%) was more common than *A. aceti*, infecting 2 must samples (11.8%) and 1 wine sample (7.8%) (Table 1, Figure 2). Both *Acetobacter* species were detected predominantly in must or wine samples, with only one grape sample (5.8%) infected with *A. pasteurianus*.

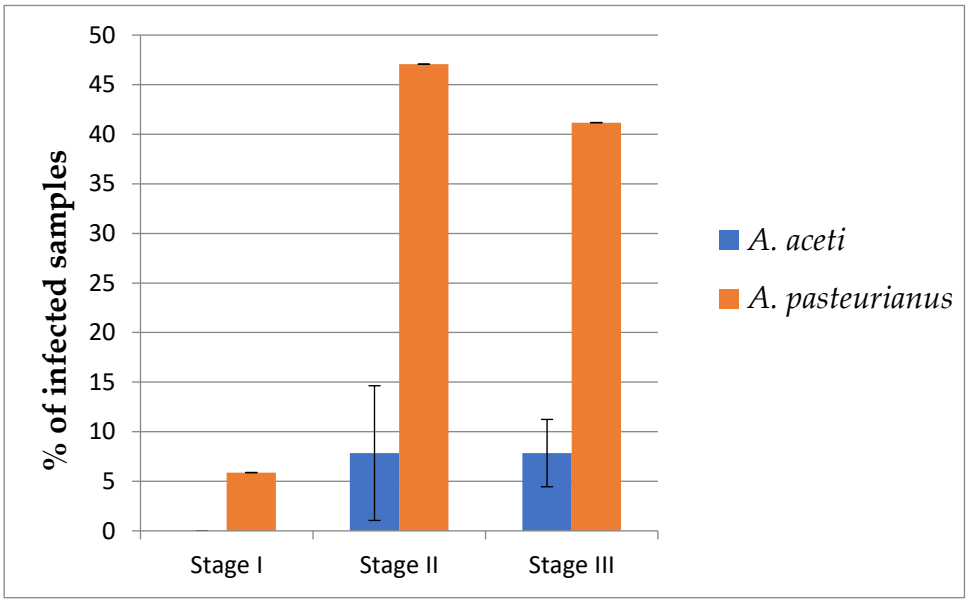

Figure 2. Percentage of samples at different stages of wine production infected with *A. aceti* or *A. pasteurianus*. For each grape variety, three samples at three stages of winemaking were analyzed by PCR. Number of samples positive for infection was counted, average and standard deviation was calculated.

A. aceti was detected at marginal value (Cq=39,18) in Feteasca Alba in only one out of three experiments, so, it resulted in high standard deviation value. Since *A. aceti* was found in only three samples at low levels (high value of Cq>33) and apparently did not have a prominent effect on wine acidity, further discusstion will be focused on *A. pasteurianus*.

Considering the stage of winemaking at which the infection occurred, only one variety (5.8%–Rara Neagra) had detectable amounts of AAB (*A. pasteurianus)* at all analyzed stages I, II, III (I – grape, II – must, III – wine). Two samples (11.7%–Viorica and Nistreana) had detectable amounts of *Acetobacter* species at two stages II, III (both must and wine). Six samples (35.2%–Ametist, Feteasca Regala-Cricova, Pinot Gris, Cabernet Petit, Feteasca Neagra-Milestii Mici (MM), Feteasca Regala-Orhei) had *A. pasteurianus* in must, and five samples (29.4%–Rkatiteli, Augustina, Alexandrina, Feteasca Regala-Cahul, Feteasca Alba-Straseni) had *A. pasteurianus* in wine (Figure 3). Three samples (Feteasca Neagra-Purcari, Feteasca Neagra-Nisporeni, Chardonnay) were negative for *Acetobacter* at all stages.

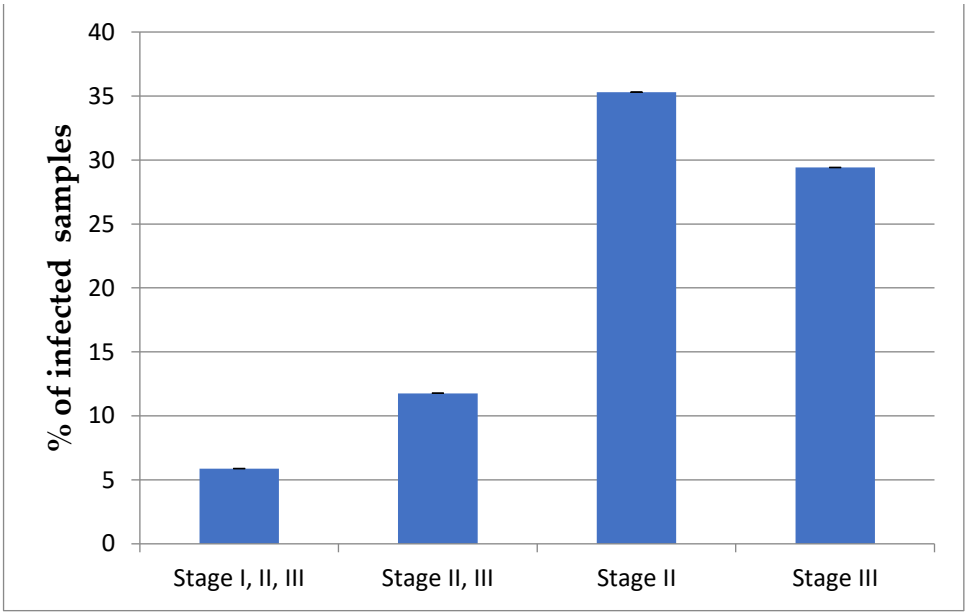

Figure 3. Percentage of wines infected with *Acetobacter* at all three stages of wine production (Stage I -wine, II - must, III - wine), two stages (Stage II - must, III - wine), or single sampling stage (Stage II - must) or (Stage III - wine). For each grape variety, three samples at three stages of winemaking were analyzed by PCR. Number of samples positive for infection was counted, average and standard deviation was calculated.

Figure 4 shows the distribution of *A. pasteurianus* in wine and must samples, expressed in the difference between Cq=40 and the actual Cq values of the samples, as well as volatile acidity of wine samples. In general, 13 out of a total of 17 samples were infected with *A. pasteurianus* at least at one stage of winemaking.

The acetic acid bacteria typically associated with grapes and must is *Gluconobacter oxydans* [3, 6, 7,]. Nonetherless, we could detect *A. pasteurianus* in 8 out of 17 analyzed must samples. Morever, some samples had a rather high content of these bacteria (Cq value about 30). This can be probably due to the fact that the must was sampled at the early stage, before active fermentation started. In two samples (Rara Neagra, Nistreana), *A. pasterurianus* is found at both Stage II – must and Stage III – wine (Figure 4A). In five samples *A. pasteurianus* is found at Stage II-must, but is not detected at the Stage III – wine. This can be explained by the previously described fact that acetic acid bacteria population is highly reduced during the must fermentation [23]. However, in this study, *A. pasteurianus* appears in the wine samples even though it had not been detected in the corresponding must samples. This is the case of Augustina, Alexandrina, Viorica, Feteasca Alba and Feteasca Regala, Cahul (Figure 4A). Interestingly, all these are white wines. A possible explanation would be that these musts were infected with a low amounts of *A. pasteurianus*, below the detection levels, and once fermentation was completed and the environment became favourable, their active growth started Alternatively, the infection could occur at winemaking site, or their active growth could be boosted by some winemaking practices [23]. Another possibility is the presence in low amount of some strains capable of surving in unfavourable fermentation conditions, who started active growth after fermentation ended.

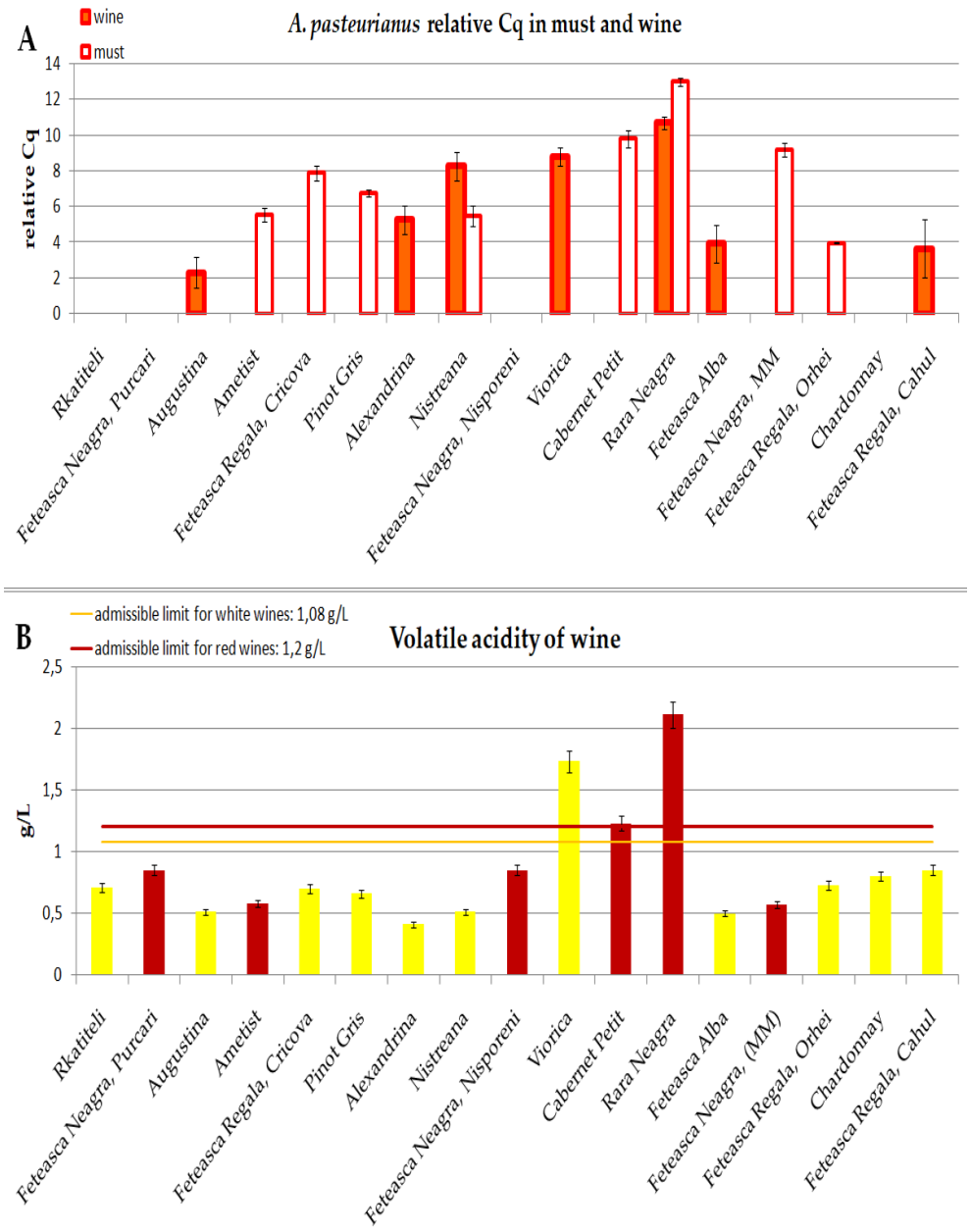

Figure 4. Distribution of *A. pasteurianus* in must and wine and volatile acidity of wine samples :.

A. Distribution of *A. pasteurianus* in must and wine, expressed as difference between Cq=40 and the actual Cq values of the samples. Average and standard deviation for three Cq values were calculated before relative Cq calculation. Wine samples are shown in red, must samples are shown in white, $p \leq 0.05$ for both must and wine samples.

B. Volatile acidity of wine samples and admissible limits expressed as acetic acid – 1.08 g/L for white wines and 1.2 g/L for red wines. Red wines are shown in dark red, white wines are shown in yellow, $p \leq 0.05$.

Rara Neagra was affected at all three stages (grape, must and wine), and also had the biggest difference of Cq value from Cq=40 in must and wine. Relatively high Cq differences were observed in Viorica (wine), Cabernet Petit and Feteasca Neagra-MM (must).

Two wine samples (Viorica and Rara Neagra) contained the most *A. pasteurianus* DNA of all samples. Since *A. pasteurianus* produce acetic acid, and acetic acid is the main

constituent of wine volatile acidity [7], the volatile acidity of the wine samples was measured.

Most wine samples had volatile acidity within the admissible limits. Volatile acidity of two wine samples (Rara Neagra and Viorica) exceeded the admissible limit. Interestingly, the same two wine samples (Rara Neagra and Viorica) had the highest content of *A. pasteurianus*. Comparing figure 4A and figure 4B, it is noticeable that the wine with highest volatile acidity, Rara Neagra, had the highest Cq value difference for *A. pasteurianus* in both wine (Cq=29,31±0,34) and must (Cq=27,02± 0,20) samples, and also was the only sample where *Acetobacter* was detected in grapes. Another wine exceeding the admissible limit for volatile acidity, Viorica, had a Cq= 31,19±0,51 (high Cq difference) in wine for *A. pasteurianus* (Figure 4A). One sample (Cabernet Petit) had marginal volatile acidity at the admissible limit. This sample had a high Cq difference (Cq=30,22± 0,48) for *A. pasteurianus* in must, but this microorganisms was not detected in wine possibly due to wine treatment or competition with other wine microorganisms.

These data suggest that *A. pasteurianus* may be at least partially responsible for increasing the volatile acidity of these wines above acceptable limits. The same conclusion was reached by the authors [24], who established that a closely related group of Acetobacter pasteurianus predominated in isolates from wines with increased volatile acidity, detected by analysis of the 16S rRNA region and RAPD-PCR Thus, *A. pasteurianus* can be considered the species responsible for the alteration [24].

Two samples (Feteasca Regala-Cricova and Feteasca Neagra -MM) though had a relatively high Cq difference of *A. pasteurianus* in must, (Cq=32,12 ± 0,41 and Cq=30,84 ± 0,40, correspondingly) but no detectable amount in wine, and thus no exceeding volatile acidity admissible limit.

4. Conclusions

In this work, we studied distribution of AAB in seventeen samples of thirteen varieties grown in three PGI regions of the Republic of Moldova at different stages of winemaking. *A. pasteurianus* was more common than *A. aceti* and also showed more prominent correlation between the relative amount of its DNA detected in wines and wine volatile acidity.

Acetobacter bacteria were not commonly found in grapes; in fact, only one grape sample had detectable amounts of *A. pasteurianus,* while *A. aceti* was not detected in any of the grape samples. This confirms previous observations that AAB genus typically associated with grapes is *Gluconobacter*.

Only one sample, Rara Neagra, was infected at all three stages of winemaking, it also had the highest relative Cq in both must and wine, and the highest volatile acidity.

Two wines (Viorica and Rara Neagra) with volatile acidity exceeding the admissible limits had also the highest relative amount of *A. pasteurianus* DNA in wine, suggesting that *A. pasteurianus* could be an important wine spoilage microorganism causing increased volatile acidity in Moldovan wines.

This research shows the perspective of PCR diagnostics for predicting the risks of wine spoilage by AAB.

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