MOLECULAR DETECTION OF ACETIC ACID BACTERIA AT DIFFERENT STAGES OF WINE PRODUCTION

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Abstract

Acetic acid bacteria (AAB) are common wine spoilage microorganisms associated with elevated levels of acetic acid in wine, which is a serious wine fault requiring winemakers' attention. Timely detection of AAB, thought essential for efficient AAB control, is however challenging due to the difficulties of their isolation. Thus, it would be advantageous to detect them using molecular methods at all stages of winemaking and storage and prevent wine spoilage. In this paper, we analyzed wines samples of varieties grown in different regions with Protected Geographical Indication of the Republic of Moldova for the presence of Acetobacter aceti and Acetobacter pasteurianus by real-time PCR. Samples were collected after clarification and stabilization, before bottling. There was observed an uneven impact of environmental factors on relative quantities of A. aceti and A. pasteurianus in red and white wines, while A. pasteurianus was the predominant species during the period of monitoring.

Keywords: *winemaking, acetic acid bacteria, wine spoilage, primers, real-time PCR*

1. INTRODUCTION

Acetic acid bacteria (AAB) is a widely distributed group of obligate aerobic bacteria well known for their ability to convert ethanol to acetic acid (Gomes et al., 2018). Current classification of AAB comprises 19 genera and 92 species with high morphological and physiological variability, *Acetobacter* being the first genus described (He et al., 2022; Qiu et al., 2021). Their ability to produce acetic acid is commonly used in vinegar production (Román-Camacho et al., 2024). However, winemakers mostly regard AAB as undesirable microbial flora since excessive biosynthesis of acetic acid causes wine spoilage (Parra et al., 2023), while controlling their propagation proves to be a challenging task, especially for small-scale wineries. It has been reported that acetic acid bacteria are capable of slow proliferation even under semi-anaerobic conditions (Bartowsky and Henschke, 2008). Therefore, common practices in wine industry that include wine blanketing with inert gas and storage in atmosphere devoid of oxygen and sulfur dioxide do not guarantee total inhibition of AAB activity (Bartowsky and Henschke, 2008; Du Toit et al., 2005).

Microbiological monitoring of *Acetobacter sp*. in wines is hindered since they can occur as viable but non-culturable (VBNC) cells (De Roos and De Vuyst, 2018). The VBNC state was described for a number of bacterial species and manifests itself as the phenomenon when microorganisms do not grow on cultural media, while remaining alive and capable of restoring their metabolic activity under natural conditions (Pazos-Rojas et al., 2023). This can result in underestimating the real levels of wine contamination with AAB during fermentation and storage. A number of researches focus on optimizing composition of media for *Acetobacter* isolation and cultivation (Guillamón and Mas, 2011). Molecular methods proved to be effective for AAB identification and monitoring since they are based on recognizing specific regions of bacterial genome and can evade certain obstacles associated to AAB

metabolism. A number of studies focused on designing and optimizing effective PCR-based assays for detecting and quantifying AAB associated with wine spoilage. qLAMP technology with primers to 16S rRNA was used for identifying *A. aceti*, while the detection limit for qPCR and qLAMP being of 2.05×10^1 CFU ml^{-1} (Zhang et al., 2020). Using RAPD-PCR technique, the *Acebacter* diversity in red wines was analyzed (Bartowsky et al., 2003). Alongside 16S rRNA regions, the heat shock protein 60 (*hsp60*) sequence was used to discriminate *Acetobacter* species (Huang et al., 2014). In winemaking, the timely detection of these microorganisms can be crucial for preventing wine spoilage and economical losses, so the development of affordable, rapid and direct methods suitable for on-site analysis is a priority. Molecular biology methods, such as quantitative PCR (qPCR), demonstrate high efficiency in the early detection and quantification of AAB and can be widely used in the winemaking process (González et al., 2006; Torija et al., 2010; Valera et al., 2013). The 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 (Gammon et al., 2007). It can also quantify PCR products with greater reproducibility while eliminating the need for post-PCR processing, thus preventing carryover contamination.

Despite having a lot of advantages, PCR detection of spoilage microorganisms have certain drawbacks. The difficulties include cell lysis and nucleic acid extraction, cross contamination and failed reactions due to the presence of inhibitory substance or competing DNA from the non-target cells (Priyanka et al., 2016). A major drawback is the inability of PCR to differentiate the DNA from dead and viable cells, and this is a critical factor for the food industry, regulatory agencies and the consumer (Zeng et al., 2016). Therefore, optimizing cultural media components and microbiological cultivation of AAB remains one of the main directions in effective monitoring of these microorganisms in winemaking (Parra et al., 2023).

Current work provides a brief survey on different factors that might affect AAB in wines of private Moldavian wineries for their possible use in monitoring the propagation of these microorganisms in wines.

2. MATERIALS AND METHODS

2.1. Wine samples

Samples of red and white wine (after clarification and stabilization, before bottling) were provided for analysis by private wineries from central and southern winemaking regions of the Republic of Moldova with Protected Geographical Indication (PGI): Valul lui Traian, Codru, Stefan Voda (Table 1). Seventeen Georgian, Romanian and international grape cultivars as well as novel local Moldavian varieties were used for producing different types of wine.

Nr.	Grape variety	PGI	Variety origin
1	Viorica	Codru	Local Moldavian variety of new selection
2	Legenda	Codru	Local Moldavian variety of new selection
3	Ametist	Codru	Local Moldavian variety of new selection
4	Nistreana	Codru	Local Moldavian variety of new selection
5	Augustina	Codru	Local Moldavian variety of new selection
6	Malena	Codru	Local Moldavian variety of new selection
7	Alexandrina	Codru	Local Moldavian variety of new selection
8	Rkatsiteli	Codru	Georgian variety
9	Saperavi	Codru	Georgian variety
10	Cabernet Petite	Codru	International variety

11	Merlot	Codru	International variety
12	Pinot Gris	Stefan Voda, Codru	International variety
13	Chardonnay	Valul lui Traian	International variety
14	Feteasca Neagra	Codru, Stefan Voda, Valul lui Traian	Local Moldavian-Romanian variety
15	Rara Neagra	Codru, Stefan Voda	Local Moldavian-Romanian variety
16	Feteasca Alba	Codru, Stefan Voda	Local Moldavian-Romanian variety
17	Feteasca Regala	Codru, Valul lui Traian	Local Moldavian-Romanian variety

Table 1. Origin of wine samples used in the study

The main operation to obtain white wines were following (Jackson, 2020): harvesting grapes; destemming and crushing grape berries; maceration at t=12–14°C for 2-4 hours, sulfuring 100–120 mg/dm3; must separation and sulphitation 50 mg\dm3; clarification of the must for 18 hours with bentonite 0.5–1.0 g/dm3 at t=10–14°C; fermentation at t=16°C for 7–10 days; post-fermentation and wine formation at t=10–12ºC; yeast removal with wine equalization and sulfuring 10–20 mg/dm3; storage; integrated wine treatment (wine fining, racking, cold treatment at t= 3–5°C for 5–7 days); cold filtration; wine resting for 10 days; membrane filtration; bottling.

The operation to obtain red wines were following (Jackson, 2020): harvesting grapes; crushing and stemming grape berries; maceration and fermentation at t=26-28°C for 4-6 days, sulfuring 75 mg/dm3; pressing grape mash; running off the free-run wine and the first fraction for additional fermentation; malolactic fermentation; wine formation on yeast lees; removal of wine from yeast lees with equalization and sulfuring (20 mg\dm3); storage; integrated wine treatment; wine fining for 10 days; racking; cold treatment at 3-5°C for 5-7 days; cold filtration; wine resting for 10 days; membrane filtration; bottling.

2.2. DNA isolation

For DNA isolation, ten mL of each wine 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, Na_2EDTA 0.025M, SDS 5 %w/v) and heated at 65°C for 1 hour. All reagents were of molecular biology grade (Sigma-Aldrich). Then, 60 mg of PVP powder and a 0.5 volume of ammonium acetate solution (7.5 M) were added to the sample and incubated on ice for 30 min. After 10-minute of centrifugation at 10000 g the supernatant was transferred to a fresh tube, mixed with equal volume of chloroform, vortexed and centrifuged again at 10000×g. 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, and the pellet washed twice with 70% ethanol, air dried and dissolved in 50 µL of water; then, 2 µL of the resulting DNA solution was used for each PCR reaction. DNA quality and concentration were checked spectrophotometrically using a Genova Nano micro-volume spectrophotometer. Three aliquotes of each sample were taken for DNA extraction.

2.3. Amplification and sequence analysis

Previously described oligonucleotides complementary to ribosomal RNA cluster and ITS sequences of *A. aceti* and *A. pasteurianus* (Priyanka et al., 2016) were used as primers for identifying the respective bacteria (Table 2).

Primer	Sequence	Species	Target-sequence (FASTA)
P173	TTTTGAAATGTGACGCGCTTGAATG	*A. aceti*	AB161358.1
P174	TTGCTCCCATGCACAGAAACC		
P175	CCGGCGGTGATCTTCTGTTC	*A. pasteurianus*	AJ888874.1
P176	CCGCTCTGTGCGTCAAACTT		

Table 2. Primers used for detecting *A. aceti* and *A. pasteurianus*

Quantitative PCR was performed via PCR detection systems CFX96 TouchTM BIORAD. Amplification protocol included 95°C for two minutes as initial denaturation step followed by alternations of 95°C for 15 sec and 60°C for 1 minute for 40 cycles. For melting curve construction, samples were heated to 95°C for 15 seconds, then incubated at 60°C for 1 minute (1.6°C/sec ramp rate), and heated to 95°C for 15 seconds (0.15°C/sec ramp rate). The detection of the amplified product was performed via the SYBR channel.

2.4. Calculation of relative Cq values

Since Cq represent the number of PCR cycles at which the fluorescent signal passes the threshold level, the value itself denotes the relative quantity of the target sequence in the sample. The higher Cq values correlate with lower copy number of DNA sequences specific for the respective microorganism to be detected and vice versa. Although comprising the necessary information on pathogen load in the sample, visualization of Cq values sometime can be cumbersome for they are inversely related to the quantity of the sequence of interest. Therefore, it is expedient to modify the respective parameter in a way it will indicate DNA quantity in direct ratio. The Cq values obtained after qPCR test were subtracted from maximum Cq values in an amplification series (Cq_{max}=41). The new data represent the comparative velocity of the fluorescent signal in different samples transiting the baseline fluorescence levels. In this way, higher relative Cq values reveal the greater initial amount of DNA of interest in the sample.

3. RESULTS AND DISCUSSIONS

There was observed a slight overall predominance of *A. pasteurianus* over *A. aceti*, both species being significant causal agents of wine spoilage. The average Cq values for *A. aceti* during the whole period of monitoring was 39.34±1.51, and for *A. pasteurianus* – 36.69±2.99. The values indicate medium to low quantities of the AAB species. However, the percentage of samples with Cq values lower than 29 (indicating a high relative content of the bacteria) was 1.75% for *A. aceti* and 10.53% for *A. pasteurianus*. Conditions of the year affected the relative content of *A. pasteurianus* in wine in a more prominent way compared to *A. aceti* (Fig.1). In addition, wine samples obtained from grapes harvested in 2022 were mostly contaminated with *A. pasteurianus*, which relative content was significantly higher compared to the respective values for *A. aceti*. The aforementioned implies that complex environmental conditions might affect AAB content on grape surface together with accumulation of carbohydrates in berries, which create different media for propagation and metabolic activity of *A. aceti* and *A. pasteurianus*.

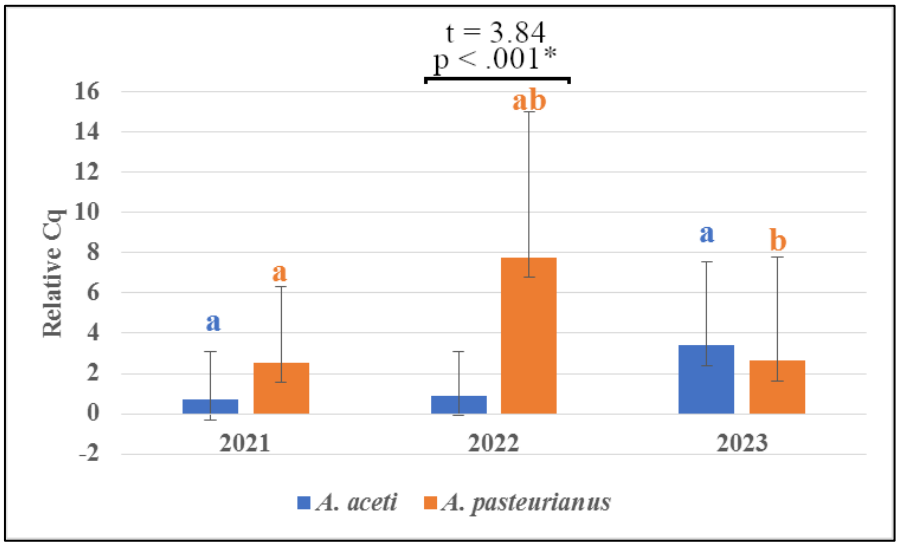

Fig. 1. Relative content of *A. aceti* and *A. pasteurianus* in wine samples obtained from grape harvests of different years. *indicates significant difference at p<.05

At the same time, mean Cq values for *A. aceti* in samples of red and white wines were 39.28±2.98 and 39.98±3.65 respectively. Again, obtained data indicate overall low content of this species, while no significant impact of wine type on accumulation of *A. aceti* was observed. Same conclusion can be stated for *A. pasteurianus*, for which the mean Cq values were 35.57±6.98 and 37.88±4.38 in white and red respectively, while the wine type did not affect the accumulation of this bacteria in the samples. Moreover, quantities of *A. aceti* and *A. pasteurianus* in red and white wines varied insignificantly regardless of the year of the harvest (Fig.2). The only exception was observed for *A. pasteurianus* in 2022 when relative quantities of this bacterium in red wines exceeded significantly the respective values in white wines. According to FAO report (FAO, 2022), in 2022 a severe drought was established in the Republic of Moldova affecting the 75% of the territory. The data suggest that some AAB species might propagate differently in red and white wines only under certain environmental conditions.

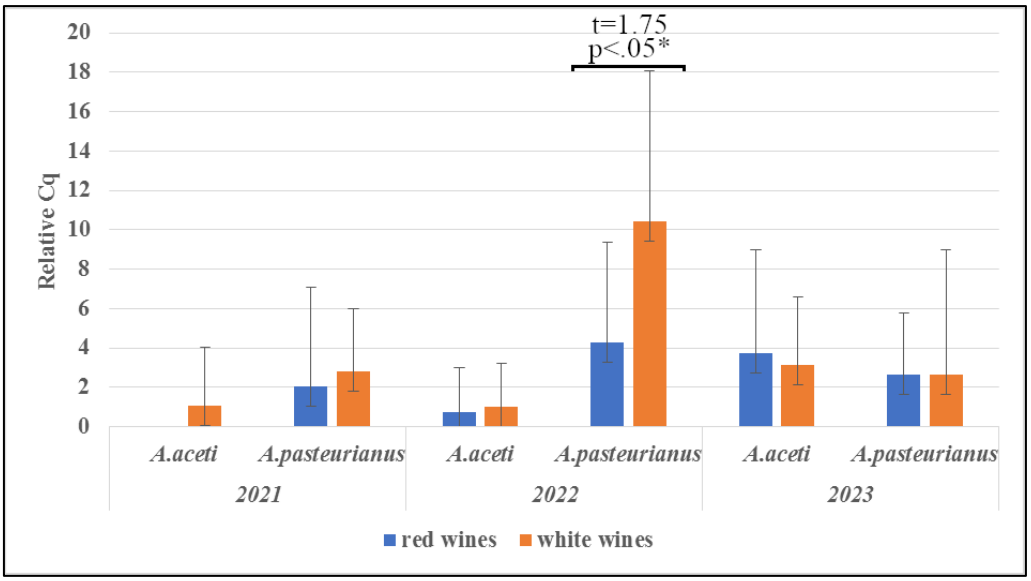

Fig. 2. Relative content of *A. aceti* and *A. pasteurianus* in samples of red and white wines obtained from grape harvests of different years.*indicates significant difference at p<.05

There was observed a difference in relative quantities of *A. aceti* and *A. pasteurianus* in wines obtained from grapes that were harvested in different regions during 2021-2023 (Fig.3). In 2021, *A. aceti* was identified in wine samples of grapes grown in Codru region, while *A. pasteurianus* was identified in wines from all three regions. In 2023 both AAB species were absent in the wine samples obtained from grapes cultivated in Stefan Voda region, while relative content of *A. aceti* was the highest in wine samples from Valul lui Traian region. In 2021 and 2022, relative content of *A. pasteurianus* was the highest in the samples obtained from Stefan Voda and Codru regions respectively. The observations imply that region of cultivation might affect in a certain way *A. aceti* and *A. pasteurianus* content in mature wine for these zones differ in soil types, average air temperatures and rainfalls. However, a limited number of wine samples, especially from southern winemaking regions does not give enough data to evaluate how significant is the impact of the region of grape cultivation on the AAB content in wine. Moreover, based on the scarce material source, it cannot be deduced to what degree do affect AAB content in wines both climatic conditions of the year and other biotic and abiotic conditions specific for certain winemaking regions.

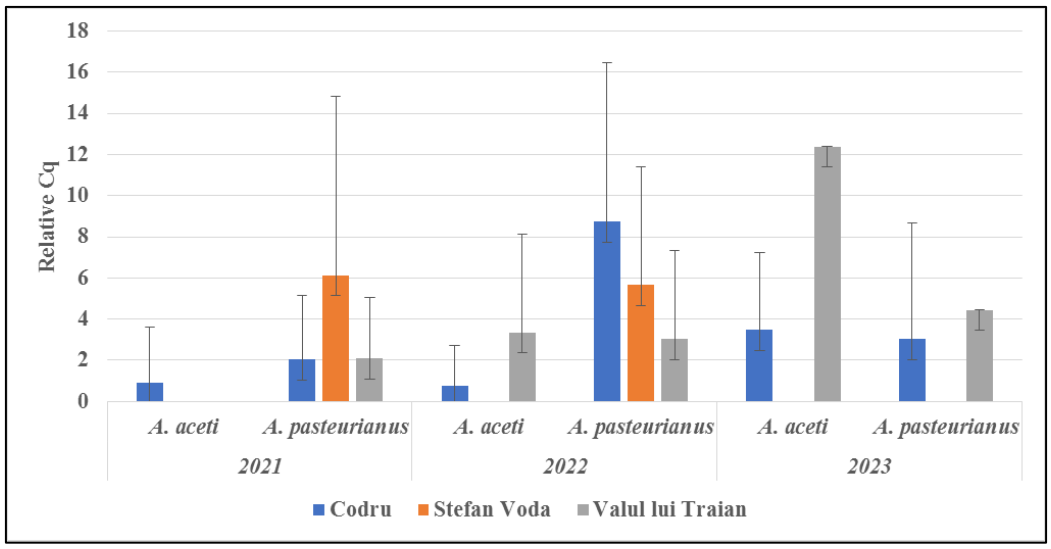

Fig. 3. Relative content of *A. aceti* and *A. pasteurianus* in wine samples obtained from grapes cultivated in different winemaking regions of the Republic of Moldova

Six grape varieties of commercial interest for Republic of Moldova were monitored regarding AAB content in mature wines obtained of these genotypes. As Figure 4 shows, most of the wines did not accumulate high contents of *A. aceti*. Wines of 'Feteasca Neagra' and 'Ametist' varieties of the two seasons were absent of the two AAB species. Wine samples of the 'Viorica' variety cultivated in 2022 and 2023 when drought was established during midseason on the major part of Moldavian territory were little contaminated with *A. pasteurianus*. Overall, relative content of the two AAB species was the highest in 'Feteasca Alba' wine samples (relative Cq=35.63±5.47), while respective parameter was the lowest for 'Feteasca Neagra' (Cq = 39.21±3.63) and 'Viorica' (Cq=37.14±5.57) wines.

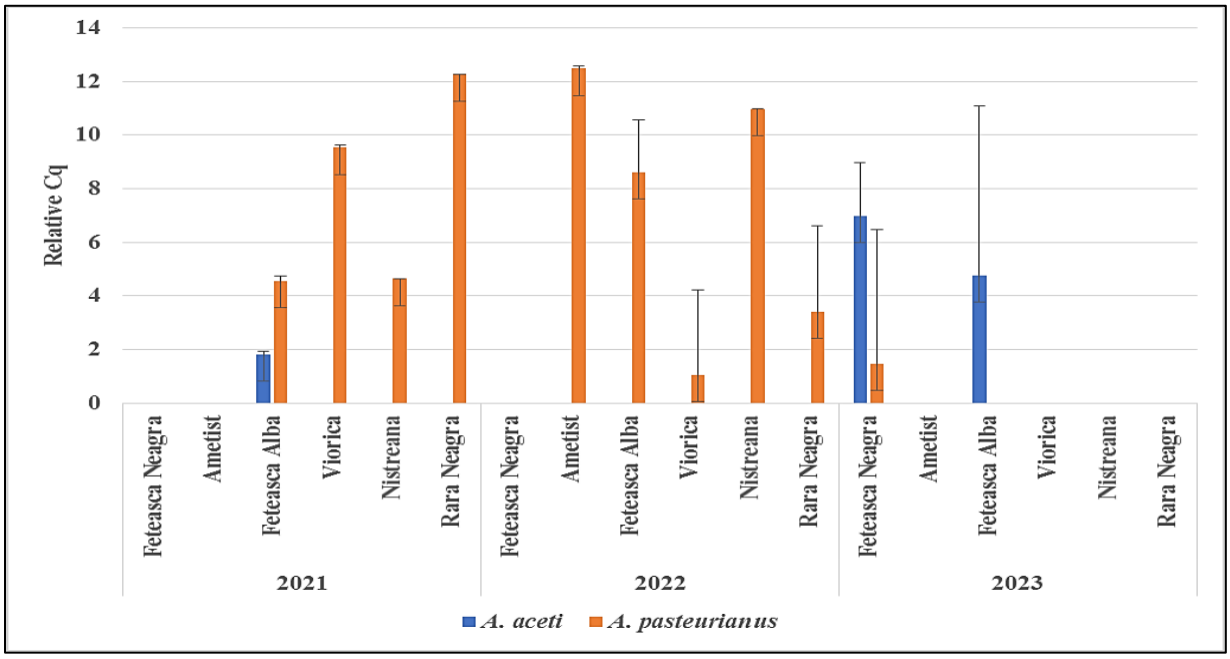

Fig. 4. Relative quantities of *A. aceti* and *A. pasteurianus* in wine samples obtained from grapes of several varieties

Therefore, it is reasonable to assume that wines produced from certain grape varieties present a more favorable media for some *Acetobacter* species due to the different content of organic compounds in berries used for fermentation. Another reason might be linked to different physiological reaction of some grape cultivars to drought conditions, the latter resulting in different accumulation of organic compounds in berries and different media for AAB propagation and activity.

4. CONCLUSIONS

AAB present a special interest for winemakers due to their ability to convert ethanol into acetic acid and thus causing wine spoilage during fermentation and storage. The relative content of A. aceti and A. pasteurianus in wine was analyzed depending on several factors associated with wine production: grape variety, climatic conditions during grape ripening and harvesting, region of grape cultivation and winemaking. Of two AAB species considered in this study, *A. pasteurianus* was more frequent in wine samples and identified in higher relative quantity compared to *A. aceti*. The obtained data demonstrate that climatic factors during grape ripening affect unevenly *A. aceti* and A. pasteurianus content in mature wine. In addition, analysis of relative Cq of the two AAB species suggests that grape varieties and region of their cultivation might affect *A. aceti* and *A. pasteurianus* content in mature wine. The results presented reveal the necessity of additional and meticulous analysis of the aforementioned factors for they can serve as indices for monitoring and forecasting the rates of wine contamination with *A. aceti* and *A. pasteurianus* during production in local Moldavian wineries.

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