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ORIGINAL RESEARCH PAPER

ACETIC ACID BACTERIA DETECTION IN WINES BY REAL-TIME PCR

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Abstract: Acetic acid bacteria (AAB) are considered one of the most common wine spoilage microorganisms. They are still difficult to cultivate on laboratory media, which highlights the importance of alternative methods of detection of these bacteria. The goal of this work was development and testing of a fast and reliable Real Time Polymerase Chain Reaction (RT-PCR)-based method for easy detection of AAB in wine. We designed two primer sets for RT-PCR for detection of AAB and compared the results obtained using these primers with those obtained using a commercial kit. The results obtained using home designed primers showed good correlation with the results, obtained with the commercial screening kit.

Keywords: *Acetic acid bacteria, detection kit, DNA, primers, real-time PCR*

INTRODUCTION

Acetic acid bacteria (AAB) are wine spoilage microorganisms and require the close attention of winemakers at all stages of wine production and storage [1]. Wine spoilage results from the ability of these microorganisms to convert ethanol into acetalaldehyde and acetic acid, production of acetoin from lactic acid, production of ethyl acetate, metabolism of glycerol to dihydroxyacetone and ropiness [2].

Two AAB genera - *Acetobacter* and *Gluconobacter which* are important in the wine industry are closely related and belong to one family [3]. The species most commonly found in wine and vinegar are: *Acetobacter aceti*, *Acetobacter pasteurianus*, *Gluconobacter oxydans*, *Gluconacetobacter hansenii*, *Gluconacetobacter liquefaciens* and *Gluconacetobacter europaeus* [4].

Acetic acid bacteria are Gram negative, rod-shaped, peritrichously or polarly flagellated when motile, mesophilic and obligate aerobes. These bacteria are ubiquitous, well adapted to high level of sugars and ethanol, are capable of oxidizing sugars, sugar alcohols and alcohols to corresponding acids [5] and can be a threat to wine-making process [6]. Besides, they seem to affect wine quality by influencing must composition and affecting the growth of yeast and lactic acid bacteria during fermentation [7]. In spite of their ubiquitous presence and their role in wine spoilage, wine associated AAB are still difficult to cultivate on laboratory media, which highlights the importance of alternative methods of detection of these bacteria [6].

Modern molecular biology methods, such as real-time PCR, demonstrate high efficiency in early detection of the potential spoilages in musts and wines and can be widely used in the winemaking process [8].

Detecting the PCR product in real-time involves the use of specific fluorescent probe (e.g., Taqman) [9] or nonspecific dye (such as SYBR Green I) [10].

Real-time PCR assay (RT-PCR) was developed using TaqMan Minor Groove Binding (MGB) probes for the specific detection and quantification of some acetic acid bacteria (AAB) (*Acetobacter pasteurianus, Acetobacter aceti, Gluconacetobacter hansenii, Gluconacetobacter europaeus,* and *Gluconobacter oxydans*) in wine and vinegar [4].

Based on the available sequences of the 16S rRNA genes, primers were developed that were successfully tested by reference strains of acetic acid bacteria. The usefulness of real-time PCR has been demonstrated by comparing the results with traditional methods (culturing, colony counting, etc.) [11]. Therefore, the real-time PCR method is fast, highly effective, sensitive and specific, which significantly improves the quality of microbiological control in the food industry [12].

MATERIALS AND METHODS

Experimental setup

Collection of samples

Grape samples were collected from different regions with Protected Geographical Indication (PGI) - *Codru* (*Nisporeni* and *Romanesti*) and *Valul lui Traian* (*Taraclia*). Grape variety samples used in this study are *Pinot-Noir*, *Merlot*, *Malbec* and *Cabernet-Sauvignon*.

Isolation of DNA from wine

Total DNA was extracted from wine samples of the following varieties: Pinot-Noir (Taraclia), Merlot (Taraclia), Merlot (Nisporeni), Malbec (Romanesti), Cabernet-Sauvignon (Taraclia).

Ten mL of each wine was centrifuged at 5000 g for 30 minutes. The pellet was resuspended in 0.6 mL of the extraction buffer (Tris-HCl 0.2M *p*H 8.0, NaCl 0.25M, Na2EDTA 0.025M, SDS 5 % w/v) and heated at 65 ºC 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 minutes 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 ºC 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 Miro-volume spectrophotometer.

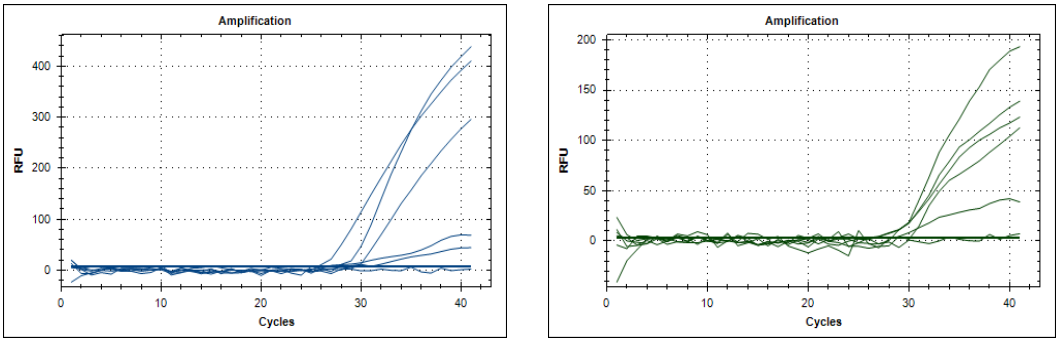
RT-PCR amplification

RT-PCR was done according to two protocols. First, the samples were analyzed using a commercial AAB screening kit 4e For everyone Detection Kit B Acetics Screening [13] according to manufacture instructions, except for the DNA isolation, which was carried out as described. Real Time-PCR was done in real-time PCR Detection Systems CFX96 TouchTM BIORAD. The PCR cycling conditions were 95 ºC for two minutes as initial denaturation step followed by alternations of 95 ºC for 15 sec and 60 ºC for 1 minute for 40 cycles. Detection of internal control was done at HEX channel, and detection of target was done at FAM channel. Then, the same samples were analyzed using home-designed primers and SYBRGreen. The PCR conditions were as recommended by SYBRGreen producer (Applied Biosystems) and met primer requirements for annealing temperature: initial incubation at 50 ºC for 2 minutes, initial denaturation at 95 ºC for 2 minutes, and alternation of 95 ºC for 15 sec. and 60 ºC for 1 minute for 40 cycles. For melting curve construction, samples were heated to 95 ºC for 15 seconds, then incubated at 60 ºC for 1 minute (1.6 ºC /sec ramp rate), then heated to 95 ºC for 15 seconds (0.15 ºC /sec ramp rate). The detection of the amplified product was done at SYBR channel.

RESULTS AND DISCUSSION

We analyzed five wine samples Pinot-Noir (Taraclia), Merlot (Taraclia), Merlot (Nisporeni), Malbec (Romanesti), Cabernet-Sauvignon (Taraclia) using a commercial AAB screening kit 4e For everyone Detection Kit B Acetics Screening. According to the manufacturer, this kit is capable of detecting the members of the acetic acid bacteria group including the well known *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* species, and also other rare occuring but also acetic acid producing representatives as *Acidisphaera* sp., *Acidocella* sp., *Acidomonas* sp., *Asaia* sp., *Granulibacter*, *Kozakia* sp. and *Swaminathania* sp. Figure 1 and Table 1 show the results of the analysis of the wine

samples using the screening kit. Figure 1a shows the amplification curves obtained for the target (FAM channel) while Figure 1b shows those obtained for the internal control (HEX channel) for the analyzed wine samples.



a ***b***

Figure 1. *Amplification curves obtained for the target (FAM channel, **a**) and internal control (HEX channel, **b**) for the analyzed wine samples*

Table 1 summarizes the Cq values obtained for the target (FAM channel) and internal control (HEX channel) for the analyzed wine samples in this experiment.

Table 1. *Cq values obtained for the target (FAM channel) and internal control (HEX channel) for the analyzed wine samples*

Wine sample	Cq of target, FAM channel	Cq of internal control, HEX channel
Pinot-Noir (Taraclia)	26.72	28.27
Merlot (Taraclia)	27.74	26.01
Merlot (Nisporeni)	31.08	28.87
Malbec (Romanesti)	25.72	39.43
Cabernet-Sauvignon (Taraclia)	29.49	22.94

As one can see, AAB were detected in all of the analyzed samples. Next, we designed primers for detection of the most common AAB: *Acetobacter aceti* and *Acetobacter pasteurianus* using Sybr Green I as a dye. Using the Primer design tool [14], for RT-PCR analysis of Acetobacter we developed two primer pairs (p171-172 and p173-p174) for detection of based on the sequence and two primer pairs (p175-176 and p177-p178) for detection of, based on the sequence of AJ888874.1 *Acetobacter pasteurianus* partial adhA gene for alcohol dehydrogenase. Primer names, sequences and characteristics are shown in Tables 2 and 3.

Table 2. *Primers for the analysis of Acetobacter aceti based on the sequence AB161358.1 Acetobacter aceti genes for 16S rRNA, 16S-23S rRNA ITS and 23S rRNA*

Primer name	Primer orientation	Sequence (5'- >3')	Length	Start	Stop	Tm	GC [%]	Self 5' complementarity	Self 3' complementarity	Product length
P171	Forward primer	TGAAATGTGACGCGCTTGAATGAG	24	723	746	62.65	45.83	4.00	1.00	92
P172	Reverse primer	TGCTCCCATGCACAGAAACCA	21	814	794	62.55	52.38	4.00	0.00	
P173	Forward primer	TTTTGAAATGTGACGCGCTTGAATG	25	720	744	62.01	40.00	5.00	2.00	96
P174	Reverse primer	TTGCTCCCATGCACAGAAACC	21	815	795	61.43	52.38	4.00	0.00	

Table 3. *Primers for the analysis of Acetobacter pasteurianus, based on the sequence AJ888874.1 Acetobacter pasteurianus partial adhA gene for alcohol dehydrogenase*

Primer name	Primer orientation	Sequence (5'->3')	Length	Start	Stop	Tm	GC [%]	Self complementarity	Self 3' complementarity	Product length
P175	Forward primer	CCGGCGGTGATCTTCTGTTC	20	347	366	61.08	60.00	5.00	0.00	100
P176	Reverse primer	CCGCTCTGTGCGTCAAACTT	20	446	427	61.50	55.00	5.00	3.00	
P177	Forward primer	CGGCTCACCAGATTCCGTTTG	21	113	133	61.86	57.14	3.00	1.00	125
P178	Reverse primer	GGTACGGGCTTCTGGGGTATC	21	237	217	61.98	61.90	4.00	2.00	

We performed BLAST analysis of these primers in GeneBank database to check their specificity and inclusivity. Table 4 shows the number of hits of these primers.

Table 4. *Blast analysis of the home-designed primer pairs*

Primer name	*A. pasteurianus*	*A. aceti*	*A. pomorum*	*A. ascendens*	*A. oryzifermentans*
P171	-	7	-	-	-
P172	-	7	-	-	-
P173	-	7	-	-	-
P174	-	7	-	-	-
P175	16	1	3	-	1
P176	28	1	3	3	2
P177	28	1	-	-	-
P178	28	1	-	-	-

As one can see from the Table 4, primers p171-p174 are specific to only *Acetobacter aceti* sequences and do not recognize other sequences. Primer pairs p175-p178 recognize *Acetobacter pasteurianus*. At the same time, all four primers designed for

Acetobacter pasteurianus recognize one nucleotide sequence of *Acetobacter aceti*. However, it should be noted that in all four cases it the same sequence (D90004.1). Besides, BLAST analysis of this sequence (D90004.1) shows its homology to alcohol dehydrogenase gene of *Acetobacter pasteurianus*. It can be related to erroneous identification of the bacterium during sequencing.

Next, we analyzed the same five wine samples with confirmed infection with AAB with home designed primers. Table 5 summarizes the results of the analysis of the samples.

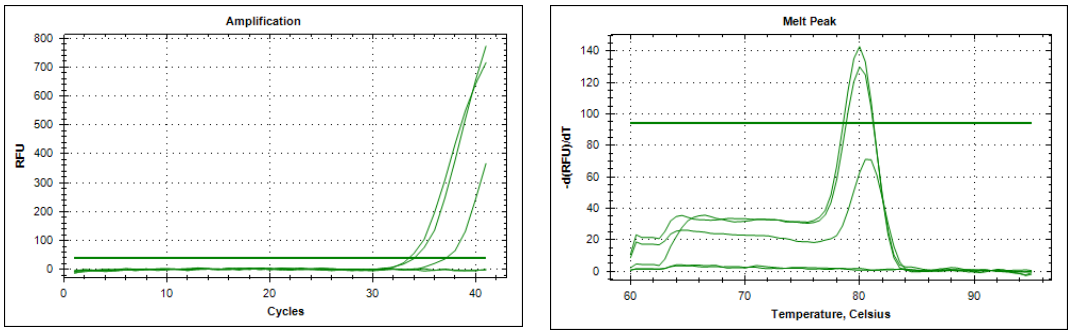
Table 5. *Cq values obtained with each of the designed primer pair for each of the analyzed samples*

Wine sample	**Cq value**			
	Acetobacter aceti		***Acetobacter pasteurianus***	
	p171-172	**p173-174**	**p175-176**	**p177-178**
Pinot-Noir (Taraclia)	-	-	-	-
Merlot (Taraclia)	-	-	31.33	32.00
Merlot (Nisporeni)	37.06	35.57	34.24	-
Malbec (Romanesti)	33.45	32.34	27.31	29.19
Cabernet-Sauvignon (Taraclia)	34.01	33.11	34.36	39.81

Analyzing the data shown in the Table 1, one can see that neither *Acetobacter aceti,* nor *Acetobacter pasteurianus* was detected in the sample Pinot-Noir (Taraclia), even though this sample was positive by AAB screening kit. It can indicate that this sample was detected by other acetic acid producing bacteria, like *Gluconacetobacter, Gluconobacter,* or some rare AAB. Merlot (Taraclia) was infected by *Acetobacter pasteurianus* (both primer pairs for detection of *Acetobacter pasteurianus* gave a positive signal), but not *Acetobacter aceti* (no signal from either of the two *Acetobacter aceti* detecting primer pairs). The samples Malbec (Romanesti) and Cabernet-Sauvignon (Taraclia) were infected with both *Acetobacter aceti* and *Acetobacter pasteurianus,* with all four primer pairs giving a positive signal. As for the sample Merlot (Nisporeni), it was infected with *Acetobacter aceti,* with both primer pairs detecting *Acetobacter aceti* giving a positive signal. Only one primer pair of the two for *Acetobacter pasteurianus* detection gave a positive signal for this kind of wine. This can be explained by the difference in primer specificity and their ability to recognize different *Acetobacter* species (discussed in more details later).

For each of the primer pairs, amplification and melt curves were analyzed. Figure 2 shows the amplification (Figure 2a) and the melt curve (Figure 2 b) obtained by the primer pair p171-172, recognizing *A. aceti*.

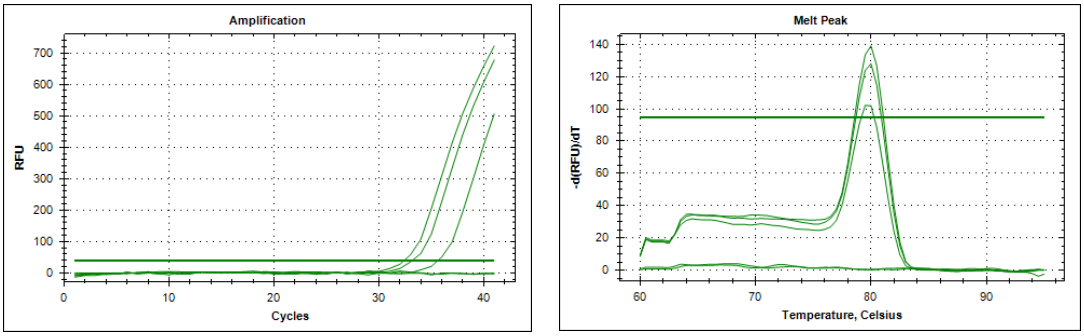
In Figure 2a, the amplification plot shows growth for the samples Merlot (Nisporeni), Malbec (Romanesti) and Cabernet-Sauvignon (Taraclia) indicating that these samples are positive for *A. aceti*, with Malbec (Romanesti) sample having the highest amount of A.aceti DNA (Cq value 33.45, Figure 2a, Table 5) and Merlot (Nisporeni) the lowest amount of *A. aceti* DNA (Cq value 37.06, Figure 2a, Table 5) of all positive samples.



a ***b***

Figure 2. *Amplification* ***(a)*** *and melt curve* ***(b)*** *obtained by the primer pair p171-172*

Similar results were obtained by the primer pair p173-174, also recognizing A. aceti (Figure 3a, Table 5). In the analysis with the primer pair p173-174, Merlot (Nisporeni) also showed the lowest amount of *A. aceti* DNA (Cq value 35.57, Figure 3a, Table 5), and Malbec (Romanesti) the highest amount of *A. aceti* DNA (Cq value 32.34, Figure 3a, Table 5) of all positive samples.



a ***b***

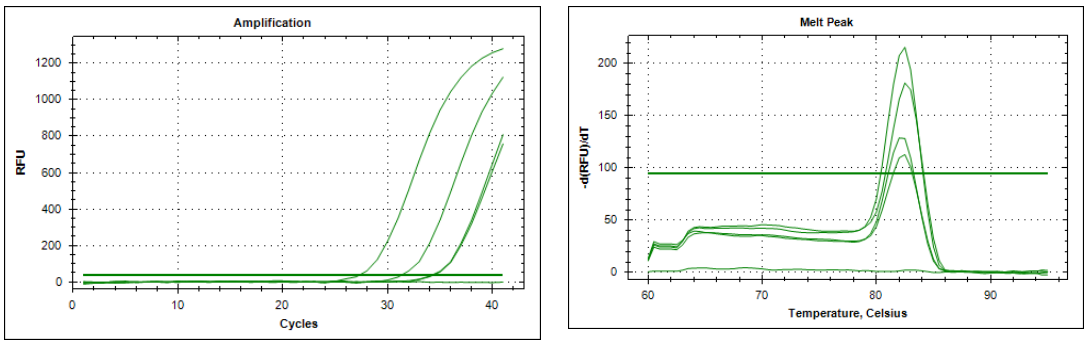
Figure 3. *Amplification* ***(a)*** *and melt curve* ***(b)*** *obtained by the primer pair p173-174*

Analyzing melting curves of the fragments amplified by the primer pair p171-172 (Figure 2b), one can see that all three amplified fragments produced a single well pronounced melt peak. However, since the amount of the A. aceti DNA in the sample of Merlot (Nisporeni) was close to the limit of detection (Cq value 37.06, Table 5), -d(RFU)/dT was below the threshold set. The melt peak temperature for the samples Malbec (Romanesti) and Cabernet-Sauvignon (Taraclia) was 80 ºC.

As for the melting curves of the fragments amplified by the primer pair p173-174 (figure 3b), we again can observe a single well pronounced melt peak produced by the fragments amplified by all three primer pairs. The melt peak temperature for the samples Malbec (Romanesti) and Cabernet-Sauvignon (Taraclia) was 80 ºC, while that for the sample Merlot (Nisporeni) was 75.5 ºC. This can be explained by the variation of the amplified sequence between different *A. aceti* stains.

Figures 4 and 5 show the graphs obtained by the primer pairs recognizing A. *pasteurianus.* Figure 4a shows that amplification plots grow for the samples Merlot (Taraclia), Merlot (Nisporeni), Malbec (Romanesti) and Cabernet-Sauvignon (Taraclia), with Malbec (Romanesti) having the highest amount of target DNA (Cq value 27.31,

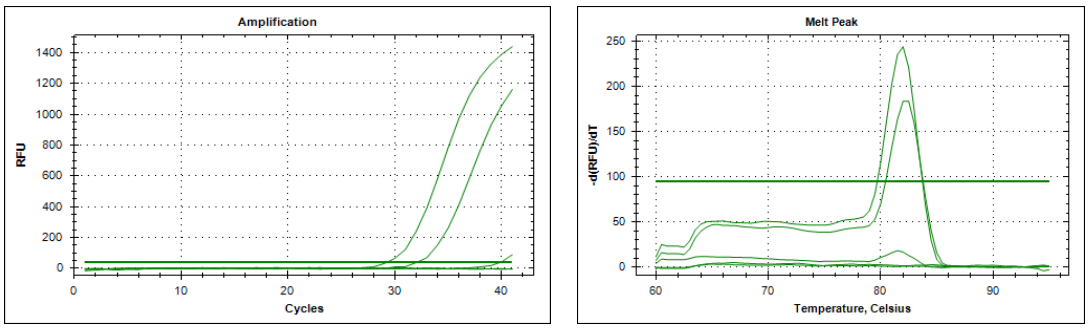
Figure 4a, Table 5), and Cabernet-Sauvignon (Taraclia) the lowest amount of target DNA (Cq value 34.36, Figure 4a, Table 5) of all positive samples.



a ***b***

Figure 4. *Amplification* ***(a)*** *and melt curve* ***(b)*** *obtained by the primer pair p175-176*

The results obtained by the primer pair p177-178, also recognizing *A. pasteurianus* DNA, (Figure 5a, Table 5) were different. In this case, the amplification plots of only two samples: Merlot (Taraclia) and Malbec (Romanesti) demonstrated positive growth, with Malbec sample having higher amount of target DNA (Cq value 29.19, Figure 5a, Table 5), and Merlot (Taraclia) lower amount (Cq value 32.00, Figure 5a, Table 5). As one can see, the two primer pairs (p175-176 and 177-178), both targeted to recognize A. *pasteurianus* DNA, produced different results, with p175-176 resulting in four positive samples out of five, and p177-178 two positive samples and one sample with inconsistent result on the limit of detection (Cabernet-Sauvignon (Taraclia), Cq value 39.81). This can be explained by different primer specificity. Analyzing Table 4, which indicates *Acetobacter* species recognized by each primer pair, one can notice, that the primer pair p175-176 is specific to *A. pasteurianus,* while primer pair p177-178 can also recognize *A. pomorum, A. ascendens* and *A. oryzifermentans.* So, the most probable interpretation of the results obtained with the primers p175-176 and p177-178 is that two of the analyzed wine samples (Merlot (Taraclia) and Malbec (Romanesti**)** are infected with *A. pasteurianus,* while Merlot (Nisporeni) and Cabernet-Sauvignon (Taraclia) are infected with a different *Acetobacter* species, recognized by the primer pair p177-78.



a ***b***

Figure 5. *Amplification* ***(a)*** *and melt curve* ***(b)*** *obtained by the primer pair p177-78*

Analyzing melting curves of the fragments amplified by the primer pair p175-176 (Figure 4b), one can observe a single well pronounced melt peak for all of the amplified fragments, with the temperature of 82.5 ºC in case of Merlot (Taraclia), Malbec (Romanesti), Cabernet-Sauvignon (Taraclia) and 82.0 ºC in case of Merlot (Nisporeni). This can be explained by the variation of the amplified sequence between different *Acetobacter* species.
Analyzing melting curves of the fragments amplified by the primer pair p177-177 (Figure 5b), one again can observe a single well pronounced melt peak for all of the amplified fragments, with the temperature of 82.50 ºC in case of Merlot (Taraclia) and 82.0 ºC in case of Malbec (Romanesti). This can be explained by the variation of the amplified sequence between different *Acetobacter pasteurianus* strains.
So, both the commercial AAB screening kit and home designed primers gave consistent information about the contamination of the wine samples with AAB. Combining the two approaches we could get some complementary information about the nature of the AAB. Using the screening kit, we could determine that all of the analyzed samples are infected with AAB. Using home-designed primer pairs, we could find out that two of the analyzed samples Malbec (Romanesti) and Cabernet-Sauvignon (Taraclia) were infected with both *A. aceti* and *A. pasteurianus.* One sample: Merlot (Taraclia) was infected with *A. pasteurianus,* and one sample: Merlot (Nisporeni) with a different *Acetobacter* species. Also, one sample: Pinot-Noir (Taraclia) was probably infected with a non-*Acetobacter* AAB species.
Thus, the combination of commercial screening kit and more specific home-designed primers can give a good insight to the possible exposure of the wine to AAB, with home-designed primers be reliable and less expensive alternative. Early detection of undesirable microorganisms in wine can help take timely measures and prevent wine spoilage.

CONCLUSIONS

In this work, we combined the use of a commercial AAB screening kit and home designed primers for detection of AAB in wine samples. It was shown that primers based on the sequences of *Acetobacter aceti* genes for 16S rRNA, 16S-23S rRNA ITS and 23S rRNA and primers based on the sequences of *Acetobacter pasteurianus* partial adhA gene for alcohol dehydrogenase can specifically amplify the target DNA fragment, and thus can be used for the analysis of wine for the presence of *Acetobacter*.
It was shown that different tools used for the analysis give complementary information, and by combination of those tools one can get an insight to the nature of the bacteria present in each sample.

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