Acetobacter aceti Fast Identification by Real Time PCR in Spoiled Wine Samples

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Abstract

Wine is a beverage made from grape berries. However, without the help of beneficial microorganisms, fermentation and producing of good wine is not possible. But very often wines contain acetic acid bacteria, which are undesirable in winemaking process. Acetic acid bacteria as known as a vinegar bacteria are Gram-negative, aerobic, rod-shaped and ubiquitous bacteria. This study was focused on species of acetic acid bacteria, specifically *Acetobacter aceti* that make spoilage in wine. The aim of our study was the identification of *Acetobacter aceti* in spoiled red wine samples, with plate dilution method on agar plates and using sensitive Real Time PCR method. We used five different red wine samples for this experiment: Alibernet 2013, Blaufränkisch 2013, Cabernet Sauvignon 2013, Dunaj 2012 and Saint-Laurent 2012. Next we extracted DNA from wine samples and from pure *Acetobacter aceti* CCM 3620^T strain. Susceptibility of *Acetobacter aceti* was varied in different isolates from 10^2 to 10^7 CFU.mL^{-1}. The number of *Acetobacter* cells on GYC medium ranged from 4.05 to 4.83 log CFU.mL^{-1} in different wine samples. The higher number of *Acetobacter* cells (4.83 log CFU.mL^{-1}) was found in Cabernet Sauvignon 2013 wine.

Keywords: *Acetobacter aceti*; Real Time PCR; Spoilage, Wine.

1. Introduction

Improving the quality of food is key requirement for the food industry. There are a number of factors which have made this area one of growing importance, including increasing health consciousness among consumers, the link between diet and health ageing population etc. Consumers views, industry initiatives and labeling regulations are changing the way food ingredients are seen in the marketplace [1].

Acetic acid bacteria (AAB) from family *Acetobacteraceae* in present days include 33 genera. The most important acetic acid bacteria for winemaking process are genus *Acetobacter*, *Gluconobacter* and *Gluconacetobacter*. Nowadays the genus *Acetobacter* contains 32 species and 11 subspecies belongs to family *Acetobacteraceae* [2]. They are ubiquitous, Gram-negative, non-sporing, catalase positive and oxidase negative, rod-shaped bacteria, independently in pairs or chains, with peritrichously flagellum [3]. In general, AAB belonging to the genus *Acetobacteracieae* been found more frequently than those belonging to *Gluconobacter* [4]. The main differences between these two genera were both cytological and physiological. The main physiological difference was that *Acetobacter* oxidized ethanol into acetic acid and, subsequently, completed the oxidation of acetic acid into water and CO_2. On the other hand, *Gluconobacter* species were unable to complete this oxidation of acetic acid [5]. AAB are involved in some important industrial process [6; 7]. On the other hand, AAB are sometimes involved in foods and beverages in detrimental way, such as in wine, beer and make spoilage [8]. They're able to carry out the oxidation of different kind of alcohols and sugars and some species have a relevant importance in vinegar production for their ability to oxidize ethanol to acetic acid [9] Wines spoiled by AAB have characteristic volatility, a vinegar-

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like sourness on the palate and a range of acetic, nutty, sherry-like, solvent or bruised apple aromas and often a reduction in fruity characters. Bacterial spoilage has also recently been reported to occur in packaged wine such as vertically upright bottles [10]. Grapes and wine are subject to spoilage by AAB at many stages during the winemaking process [11].

The aim of our study was the identification of *Acetobacter aceti* in spoiled red wine samples, with plate dilution method on agar plates and using sensitive Real Time PCR (qPCR) method.

2. Materials and methods

Microbial parameters were collected after wine bottling. We collected the data from these five wine samples: Alibernet 2013 (dry), Blaufränkisch 2013 (dry), Cabernet Sauvignon 2013(dry), Dunaj 2012 (dry) and Saint-Laurent 2012 (dry). For plate dilution method we used 1 mL of wine sample added into the 99 mL physiological saline (0.9% NaCl), that represent dilution 10^{-2} and from 10^{-2} dilution added 1 mL of diluted wine into the 9 mL of physiological saline that represent dilution 10^{-3}.

Cultivation of Acetobacter aceti: We cultivated pure *Acetobacter aceti* culture (control) on Glucose Yeast extract Calcium carbonate agar (GYC) (Conda, Spain) at 30°C, 48h. GYC agar contains: 50 $g.L^{-1}$ glucose, 10 $g.L^{-1}$ yeast extract, 5 $g.L^{-1}$ $CaCO_3$ and 20 $g.L^{-1}$ agar. Final pH is 6.8 ± 0.2 at 25°C. On the same agar medium we determinate the of colony forming unit (CFU) counts in wine samples by plate diluting method, what means the total *Acetobacter* cells in 1 mL of wine sample. GYC agar described by Swings [12] detects the presence of acid-producing microorganisms and is regarded as "standard growth medium" for acetic acid bacteria.

Bacterial strain: The *Acetobacter aceti* strain used in this study was follow: *Acetobacter aceti* CCM 3620^T purchased from Czech Collection of Microorganisms in Brno.

DNA extraction: For DNA extraction we used GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich, UK). We extracted DNA from pure bacterial cell culture after 48h incubation on GYC agar, and from wine samples, which incubated for one week at 28°C aerobically. The extraction procedure for the wine samples the same that for the pure bacterial culture, but in wine samples firstly centrifuged and washed the pellet with 0.9% NaCl twice. We harvested bacterial cell culture from the Petri dish with sterile inoculation loop. The cell culture was transferred into the 2 mL Eppendorf tube with 200 µL of Resuspension Solution. Next we added 20 µL Proteinase K and 200 µL Lysis Solution C, vortex thoroughly about 15 second and incubate at 70°C during 10 min. For Column preparation we added 500 µL Column Preparation Solution to each GenElute™ Miniprep Binding Column (BC), and centrifuged at 12,000 x g for 1 min. After incubation 200 µL of ethanol (96-100%) were added in the lysate vortex thoroughly 5-10 sec. homogeneous solution is essential. Transfer the entire contents of the tube into the treated binding column and centrifuge at 6,500 x g for 1 minute. Discard the collection tube containing the flow-through liquid and place the binding column in a new 2 mL collection tube For first washing Added 500 mL of Wash Solution to the binding column and centrifuge for 1 minute at 6,500 x g. Discard the collection tube containing the flow-through liquid and place the binding column in a new 2 mL collection tube. Followed second wash added another 500 mL of Wash Solution to the binding column; centrifuge for 3 minutes at maximum speed (12,000-16,000 x g) to dry the binding column. The final step was DNA elution and 200 µL of Elution solution directly to the center of the BC membrane was added, then centrifuged for 1 min. at 6,500 x g. Incubated the DNA at room temperature for 5 min. Stored the DNA in freezer at -20°C.

Real Time PCR (qPCR) and primers: After DNA extraction we were prepared the samples for quantitative Real Time PCR. We used SensiFAST™ SYBR Hi-ROX kit (Bioline, Germany) with passive reference ROX dye, 0.8µL of each primer (Sigma Aldrich), 6.40µL ultra-pure H_2O and 2µL DNA extracted from bacterial cell culture and wine samples after incubation. We used 3-step cycling (45 cycles): Polymerase activation 2 min at 95ºC, denaturation 10 sec. at 95ºC, annealing 20 sec. at 66ºC and extension 40 sec at 72ºC. Melt Curve stage 15 sec. at 95ºC and 1 min. at 66ºC and 15 sec. at 95ºC. We used StepOne™ Thermal cycler from Applied

Biosystems®. We used these specific primers for *Acetobacter aceti* with 22bp. Name: Aceti-F 5´ TGGAGCATGTGGTTTAATTCGA 3´; Aceti-R 5´ GCGGGAAATATCCATCTCTGAA 3´. PCR product for *A. aceti* has 88bp. Data analysis was carried out using StepOne™ software v2.1. We used PCR plates with 48-well. 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. Standard curve were created by plotting the threshold cycle (C_t) of the qPCR, performed on dilution series of *Acetobacter aceti* DNA four times (from 10^6 to 10^3 cells mL^{-1}).

3. Results and discussion

Acetobacter aceti was found in red wines which have been exposed to oxygen and higher temperature 28°C. The **Table 1** shows the results from plate diluting method. We cannot know whether it is *Acetobacter aceti* on the Petri dishes. The grown colonies were markered as an *Acetobacter* spp. For better identification used the pure *Acetobacter aceti* culture.

Table 1. Number of *Acetobacter* cells in log CFU.mL^{-1}

Type of wine		*Acetobacter* cells
Alibernet	1.	4.05
	2.	4.18
	3.	4.13
Cabernet Sauvignon	1.	4.41
	2.	4.50
	3.	4.83
Blaufränkisch	1.	4.16
	2.	4.20
	3	4.12
Dunaj	1.	4.26
	2.	4.48
	3.	4.67
Saint-Laurent	1.	4.22
	2.	4.17
	3.	4.39

The number of *Acetobacter* cells on GYC medium ranged from 4.05 to 4.83 log CFU.mL^{-1} in different wine samples. The higher number of *Acetobacter* cells (4.83 log CFU.mL^{-1}) was found in Cabernet Sauvignon 2013 wine. Acetic acid bacteria include *Acetobacter aceti* are mesophilic microorganisms and their optimum growth temperature is between 25 and 30°C [13]. Enumeration, isolation, identification and preservation of AAB are not easy. Not all the media support growth of AAB equally and they are selective for one strain to another [14].

After Real Time PCR we have results include Amplification plot and Melt analysis to us precisely specify that it is *Acetobacter aceti*. The detection limit of *Acetobacter aceti* ranged from 10^6 to 10^3 cells in diluted DNA. The detection limit of pure DNA extracted from pure *Acetobacter aceti* represent 10^7 cells mL^{-1}. In wine samples the detection limit was equals or less than 10^2 cells in mL^{-1}. The threshold cycle (C_t) for pure *Acetobacter aceti* DNA was in the 19.46 cycle, 3 times diluted DNA (30µL DNA to 60µL ultra-pure H_2O). First dilution: C_t was in 20.93 cycle, second dilution: C_t was in 23.18 cycle, third dilution: C_t was in 24.09 cycle and the last dilution represent in 26.21 threshold cycle. The evaluation of threshold cycle for red wine samples we can see on **Table 2**. The number of cycles at which the fluorescence exceeds the threshold is called the threshold cycle (C_t) or, according to the MIQE guidelines, quantification cycle (C_q) [15].

Table 2. Threshold cycle of DNA from wine

DNA from wine	Threshold cycle (Ct)
Alibernet	36.67
Cabernet Sauvignon	32.37
Blaufränkisch	33.77
Dunaj	33.74
Saint-Laurent	35.22

Progress of quantitative real-time PCR (qPCR) graphically illustrates the amplification plot. The amplification plot is the level of dependence of the detected fluorescence (y-axis) and time, respectively cycle (x-axis). **Figure 1** shows, as Rn is plotted against PCR cycle number.

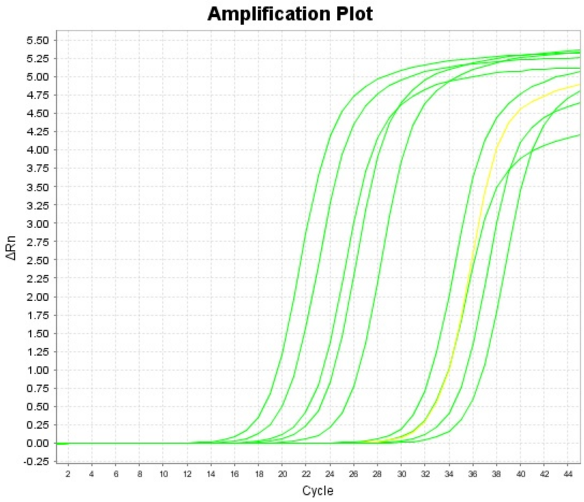

Figure 1. Evaluation of qPCR in cells of *Acetobacter*

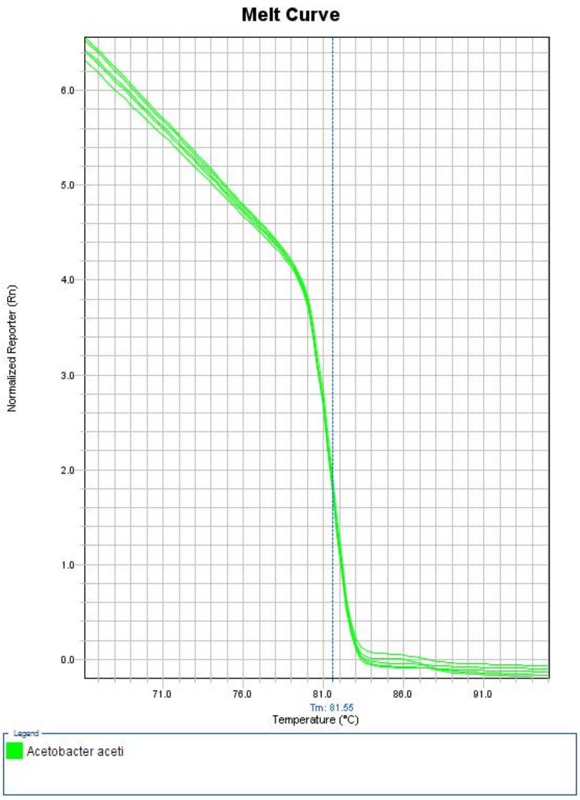

Figure 2. Standard Melt curve of *Acetobacter aceti*

Acetobacter aceti is representing the first five amplification curves from 19.46 C_t to 26.21 C_t threshold cycle with 81.55°C melting temperature (T_m) single peak. The standard melt curve (normalized reporter Rn) for *Acetobacter aceti* show in **Figure 2** and the derivative melt curve (derivative reporter -Rn) show in **Figure 3**. The next five amplification curves view one major PCR product from wine DNA in four wine samples: Alibernet, Cabernet Sauvignon, Dunaj and Saint-Laurent shown single peak, but in Blaufränkisch wine was multiple peak. There is one small contamination (**Figure 4**). We can see as a yellow amplification curve on figure 1. The most obvious situation where contamination can occur is with the transfer of specimen from DNA extraction tube to the the PCR micro-tube. Care must be taken to avoid contamination of the pipette device with specimen and to avoid the creation of an aerosol by blowing out the specimen from the tip [16]. ΔRn is the normalization of Rn obtained by subtracting the baseline: (ΔRn = Rn - baseline) The Rn (normalized reporter signal) is the ratio of the fluorescence emission intensity of the reporter dye (SYBR® Green) to the fluorescence emission intensity of the passive reference dye. Threshold is a level of ΔRn, used for the determination of the threshold cycle (C_t) in real-time assays. It is the line whose intersection with the amplification plot defines the C_t. Amplification plot shows the variation of log (ΔRn) with number of PCR cycle (**Figure 5**).

The quantitative real-time PCR method circumvents the problem of culturing AAB, and has been successfully used for the identification and quantification of AAB in grapes, wine and vinegars [17; 18]. Contrarily, acetic acid bacteria are stimulated by berry damage, increasing from less than 10 $CFU.mL^{-1}$ to 10^6 $CFU.mL^{-1}$ on rotten grapes [19]. On the contrary, Nisiotou et al. [20] found comparable acetic acid bacterial counts in soil and rotten grapes, ranging from 10^5 to about 10^6 $CFU.mL^{-1}$.

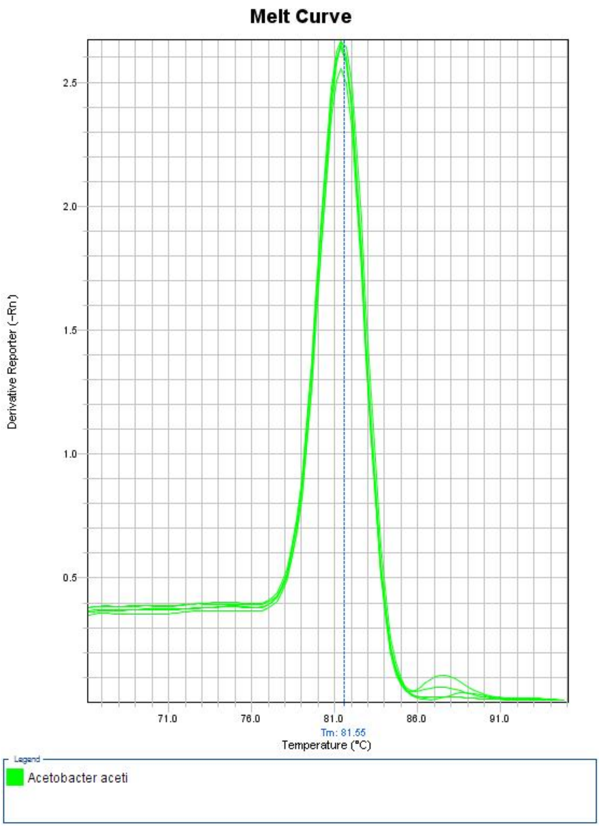

Figure 3. Derivative Melt curve of *Acetobacter aceti*

AAB populations can increase to spoil grape must by the production of acetic acid. During alcoholic fermentation, however, the population of AAB tends to decrease and can fall to below 10^2 cells.mL^{-1} by the end of fermentation [11, 24]. *Acetobacter* species are commonly detected on grapes, in wine and in vinegar, with *A. aceti* and *A. pasteurianus* being the most abundant species. However, the detection of these two species has recently decreased, while the reported detection of other *Acetobacter* species has increased, including *Acetobacter oeni* in wine [21], *Acetobacter cerevisiae* and *Acetobacter malorum* on grapes and vinegar [22; 23]

The melting temperature was equal for *Acetobacter aceti*, which shown in standard and derivative melt curve, but different at wine samples. The **Table 3** had shown the melting temperature of PCR product for wine samples.

The melting analysis is very important for this type of analysis, when we had positive control as a pure *A. aceti* culture and the wine samples was incubated at 28°C for a week. This temperature include among optimal temperature for grow *Acetobacter* species in wine. Oxygen content for growth these bacteria are important. The multiple peaks at wine sample Blaufrankisch and the other wine samples show in the standard melt curve on **Figure 6**. We can see on the figure 6 the highest melting temperature belonging to Saint-Laurent wine sample. The multiple peak was marked with grey arrow. Legend of melt curve shows (A, B, C, D, E and F) different coloured rows on PCR well plate.

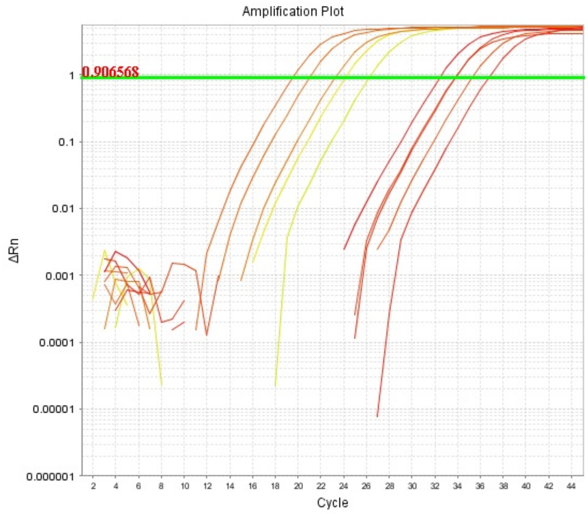

Figure 5 Amplification plot with threshold 0.906568 C_t value

Table 3 Melting temperature of wine samples

Wine sample	Melting temperature (Tm)
Alibernet	80.95 °C / Sp*
Cabernet Sauvignon	81.25 °C / Sp*
Blaufränkisch	80.65 °C / Mp*
Dunaj	80.65 °C / Sp*
Saint-Laurent	81.85 °C / Sp*

*Sp – Single peak, * Mp – Multiple peak.

Acetic acid bacteria are fastidious bacteria and can be difficult to isolate and cultivate on agar medium, despite the great number of growth media proposed. Those AAB which have proliferated in bottled wine have proven at times to be more difficult to isolate and cultivate [10].

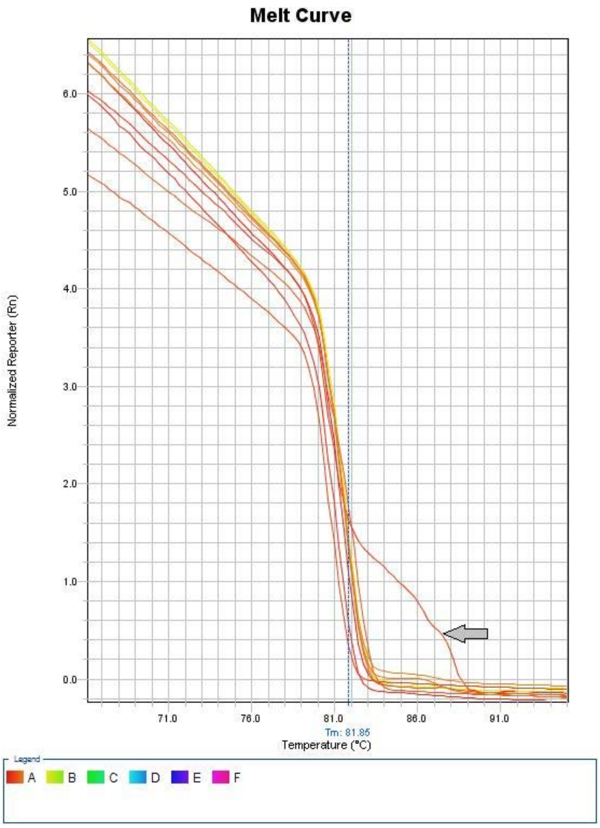

Figure 6 Standard Melt curve of wine samples

4. Conclusions

Although *Acetobacter aceti* as known as a vinegar bacteria or bacteria that cause most spoilage in must and wine, sometimes is not easy the cultivation on agar plates from wine samples. Pure *Acetobacter aceti* culture grows on different agar media for these bacteria very well. The number of *Acetobacter* cells on GYC medium ranged from 4.05 to 4.83 log CFU.mL^{-1} in different wine samples. The higher number of *Acetobacter* cells (4.83 log CFU.mL^{-1}) was found in Cabernet Sauvignon 2013 wine. The sensitivity, or detection limit, of our qPCR was fluctuating from 10^2 to 10^7 cells.mL^{-1} in all samples include DNA extracted from wine and *Acetobacter aceti*. The threshold cycles begins from 19.46 C_t to 36.67 C_t. Threshold cycle for wine samples represent more than >30 C_t and means late amplification with few amplicons. Melting temperature of *Acetobacter aceti* was 81.55°C and for other samples varied from 80.65 to 81.85°C. Wine samples Blaufränkisch and Dunaj had the same melting temperature (T_m) 80.65°C that represent the same PCR product.

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