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APPLICATION OF RT-PCR FOR *ACETOBACTER* SPECIES DETECTION IN RED WINE

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ABSTRACT

Acetic acid bacteria play a negative role in wine making because they increase the volatile acidity of wines. They can survive in the various phases of alcoholic fermentation and it is very important to control their presence and ulterior development. The main objective of the present work is to test fast, sensitive and reliable technique such as real-time PCR (rt-PCR) and detecting the presence of *Acetobacter aceti, Acetobacter pasteurianus, Gluconobacter oxydans, Gluconacetobacter liquefaciens* and *Gluconacetobacter hansenii* in red wine. The aim of our study was the identification of some species of acetic acid bacteria in red wine during the fermentation process using a classical microbiological method. The changes in different groups of microorganisms were monitored in total counts of bacteria, and *Acetobacter* cells. Microbiological parameters were observed during the current collection and processing of wine in 2012. Samples (Modry Portugal, MP and Frankovka modra, FM) were taken during the fermentation process in wine enterprises and were storage with different conditions. The total counts of bacteria ranged from 4.21 in the wine MP at 4°C of storage to 5.81 log CFU.mL^{-1} in the wine MP at 25°C of storage, but the total counts of bacteria ranged from 4.85 in the wine FM at 4°C of storage to 5.63 log CFU.mL^{-1} in the wine FM at 25°C of storage. The higher number of *Acetobacter* cells was found in wine MP at 25°C.

Keywords: red wine, fermentation process, Real time PCR, acetic acid bacteria

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 **(Horská et al., 2012)**.

Acetic acid bacteria (AAB) belong to the family *Acetobacteriaceae* and are commonly known as the vinegar bacteria. AAB are Gram$^-$, aerobic, catalase-positive microorganisms and can utilise glucose, with acetic acid as the end product **(Holt et at. 1994)**.

AAB are microorganisms 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 **(Trček and Teuber, 2002)**.

In the food industry AAB are being used as main participants in the production of several foods and beverages, such as vinegar, cocoa, kombucha and other similar fermented beverages. However, their presence and activity can easily derive into spoilage of other foods or beverages such as wine, beer, sweet drinks and fruits **(Deppenmeier et al. 2002)**.

Grapes and wine are subject to spoilage by AAB at many stages during the winemaking process **(Drysdale and Fleet, 1988)**.

As the AAB are specialised in rapid oxidation of sugars or alcohols, oxygen availability plays a pivotal role in their growth and activity. Their metabolic activity and growth is especially enhanced when oxygen is present or specifically added (for example in vinegar production). Their optimal pH is 5.5–6.3 **(De Ley et al. 1984)**.

However, they can survive and grow in the pH of the wine which can be as low as 3.0–4.0 **(Du Toit and Pretorius 2002)**.

Finally, the optimal growth temperature is 25-30°C **(De Ley et al. 1984)**, yet some strains can grow very slowly at 10°C **(Joyeux et al. 1984)**.

The most commonly species found in wine and vinegar are: *Acetobacter aceti*, *Acetobacter pasteurianus, Gluconobacter oxydans, Gluconacetobacter hansenii, Gluconacetobacter liquefaciens* and *Gluconacetobacter europaeus* **(Torija et al., 2010)**.

Gluconacetobacter europaeus is one of the most prominent AAB species isolated from industrial submerged vinegar fermentors, with high resistance to acetic acid (more than 18%) **(Sievers et al., 1992)**.

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 **(Silva et al., 2006)**.

Wines spoiled by AAB have characteristic volatility, a vinegar-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 **(Bartowsky et al., 2003)**.

AAB growth can also occur in grape must or during stuck fermentation if exposed to the air. Most commonly wines are spoiled with AAB during maturation or storage when unintentionally exposed to air **(Joyeux et al., 1984)**.

Bacterial spoilage has also recently been reported to occur in packaged wine such as vertically upright bottles **(Bartowsky et al., 2003)**.

Prevention of AAB proliferation and wine spoilage is based on an understanding that these bacteria are aerobic in their physiology and require oxygen for growth. Such growth can be prevented by practices that include blanketing wine with an inert gas such as carbon dioxide, and ensuring that storage containers are completely filled with wine to minimize contact with the headspace of air. However, it has become evident that these bacteria may survive and even multiply, albeit slowly, under semi-anaerobic conditions, such as wine stored in tanks and barrel **(Drysdale and Fleet, 1988, Joyeux et al., 1984)**.

So the presence and growth of AAB in must and wine will depend on the concentrations of SO_2. Only the molecular SO_2 has antimicrobial effects. The proportion of molecular SO_2 represents from 1% to 10% of the free form depending on the pH of the wine, therefore, the lower pH is, the higher proportion of molecular SO_2 will exist, and the higher antibacterial effect **(Ribéreau-Gayon et al., 2000)**.

The objectives of this study were to investigate the occurrence selected species of acetic acid bacteria in two different Slovakian red wines during fermentation

process and to identify the dominant acetic acid bacteria strains with Real time PCR method.

MATERIAL AND METHODS

Microbiological parameters were observed during the current collection and processing of grapes in the year 2012. Samples were taken during the fermentation process in wine enterprises. During this period were examined 4 bottles of wine in week interval among two varieties of Frankovka modra (FM) and Modry Portugal (MP). The bottles were storage at two different temperatures first at 4°C in refrigerator and second at 25°C at room temperature. The wine Frankovka modra had content 12% ethylalcohol, 2.1% sugar, 4.6% total acids, 25.4 $g.L^{-1}$ extract and 18.57 $mg.L^{-1}$ SO_2. Modry Portugal had content 11% alcohol, 1.5% sugar, 5.05% total acids, 24.7 $g.L^{-1}$ extract and 23.85 $mg.L^{-1}$ SO_2.

Determination of CFU counts

For microbiological analysis the wine samples were processed immediately after collection. The total counts of bacteria (TBC), and number of *Acetobacter* cells (A) were assessed. 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, A) in three replications. Homogenized samples of wine were prepared in advance by sequential diluting based on decimal dilution system application. For microorganism cultivation three types of cultivating mediums were used, to segregate individual microorganism groups. Glucose Tryptone Yeats agar was used for CFU segregation of TBC (incubation 72 h at 30°C, aerobic cultivation method). *Acetobacter aceti* and *Acetobacter pasteurianus* best grown in Mannitol broth media. We used 10 mL mannitol broth to bacteria species cultivation for 48 h at 30°C. *Acetobacter-Gluconobacter* agar was used for CFU segregation of *Acetobacter* cells (incubation 24-48 h at 30°C). The pure bacterial culture activation in Mannitol media and next inoculated from 1 mL of cell culture to the sterile wine samples. Cultivating medium composition corresponded to producer introductions (Biomark™, 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 shaking machine (30 minutes). Prepared basic substance was diluted to reduce the content of microorganisms below 300 CFU level.

Bacterial Strains

The AAB reference strains used in this study were as follows: *Acetobacter aceti* CCM 3620^T, *Acetobacter pasteurianus* CCM 3614, *Gluconacetobacter liquefaciens* CCM 3621^T, *Gluconacetobacter hansenii* CCM 1808, *Gluconobacter oxydans* CCM 3618. We purchased the pure cultures of bacterial strains from Czech Collection of Microorganisms in Brno.

DNA Extraction

We used two different methods for DNA extraction from bacterial cells.
The first method was very easy: Cells from bacterial cultures were harvested by centrifugation for 5 min. at 10 000 x g and washed with 1 mL of 1 M NaCl twice. The pellet than resuspended in 1 mL water, heated at 105°C and used for PCR analysis.
But for better DNA extraction we used GenElute™ Genomic DNA Miniprep Kit (Sigma Aldrich, UK).
We used procedure for Cultured Cell Preparation:
Acetobacter species are Gram$^-$ bacteria. The first step was harvested of 48 hours bacterial sample from cell culture. Putt the cell culture to the 2 mL eppendorf tube. Bacterial culture was centrifuged during 5 min / 6.000 to 10.000 g. The supernatants were removed. The pellet was dissolved in 200 µL Resuspension Solution and incubated 2 min. at room temperature. Next were added 20 µL Proteinase K and 200 µL and Lysis Solution C, vortex about 15 second and during 10 min. in 55ºC were incubated. We added 500 µL Column Preparation Solution to each GenElute Miniprep Binding Column (BC), and centrifuged at 12 000 g for 1 min. 200 µL of ethanol (95-100%) were added in the lysate and vortex mixed 5-10 sec. then about 6 500 g centrifuged for 1 min. The filtrate were removed and transferred the BC to the new eppendorf tube. Next 500 µL washing buffer was added, then centrifuged at maximum speed unless drying of membrane and then transferred to a new eppendorf tube. The washing buffer used twice. DNA elution: 200 µL of Elution solution directly to the center of the membrane was added, then centrifuged for 1 min. at 6 500 g. The purified DNA was eluted from columns with 150 mL buffer AE and stored at -20°C.

Primers and Real Time PCR

After DNA extraction we were prepared the samples for Real Time PCR. We used Sensifast SYBR Green Hi-Rox kit, specific forward and reverse primer, ultra-pure H_2O and DNA extracted from bacterial samples.

Steps of Real Time PCR: We used 3-step cycling (40 cycles): Polymerase activation 2 min at 95ºC, denaturation 5 sec. at 95ºC, annealing 10 sec. at 60ºC and extension 5 sec at. 72ºC. Melt Curve stage 15 sec. at 95ºC and 1 min. at 60ºC. We used Step One™ Thermal cycler from Applied Biosystems®.

Primers are follows:

Acetobacter pasteurianus (130 bp)	
PASTEU-F	TCAAGTCCTCATGGCCCTTATG
PASTEU-R	TCGAGTTGCAGAGTGCAATCC
Acetobacter aceti (88 bp)	
ACETI-F	TGGAGCATGTGGTTTAATTCGA
ACETI-R	GCGGGAAATATCCATCTCTGAA
Gluconacetobacter liquefaciens (98 bp)	
LIQU-F	GGGTTAAGTCCCGCAACGA
LIQU-R	ACCTTCCTCCGGCTTGTCA
Gluconobacter oxydans (122 bp)	
OXYD-F	CCCAGTGTAGAGGTGAAATTCGT
OXYD-R	CCAGGGTATCTAATCCTGTTTGCT
Gluconacetobacter hansenii (76 bp)	
HANSEN-F	GTCCACGCTGTAAACGATGTGT
HANSEN-R	TGTGCTTATCGCGTTAACTACGA

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 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 were used.

RESULTS AND DISCUSSION

Acetic acid bacteria had long been believed to play little, if any, role during winemaking operations due to their aerobic nature. Winemaking in general is an anaerobic process and the growth and survival of these bacteria under this and other unfavorable conditions like high ethanol concentrations, low pH and high SO_2 concentrations seems unlikely. Some studies have, however, shown that acetic acid bacteria can survive during fermentation and the following operations in the winemaking process, such as malolactic fermentation and during maturation of the wine. Acetic acid bacteria had been shown to contribute significantly to volatile acidity in must and wine, and the production of acetic acid by these bacteria may thus also contribute to sluggish or stuck fermentations **(Du Toit and Lambrechts, 2002).**
From microbiological parameters in wine samples total counts of bacteria and number of *Acetobacter* cell were monitored. We also monitored using real time polymerase chain reaction (RTQ PCR) qualitative representation of individual species of microorganisms from the wine samples during fermentation. Table 1 shows the number of total counts of bacteria and number of *Acetobacter* cells in MP and FM samples which were collected from wine after three weeks of storage in different temperature.
The results of microorganisms number in red wine are in table 1. The total number of bacteria ranged from 4.21 to 5.81 log $CFU.mL^{-1}$, the number of *Acetobacter* cells ranged from 4.12 to 4.73 log $CFU.mL^{-1}$. The higher number of total count of bacteria was found in MP at 25°C of storage. The higher number of *Acetobacter* cells (4.73 log $CFU.mL^{-1}$) was found in MP wine at 25°C of storage.

Table 1 Number of microorganisms in log $CFU.mL^{-1}$

Type of wine		TCB	A
Modry Portugal at 4°C	**1.**	4.89	4.16
	2.	4.21	4.12
	3.	5.64	4.27
Modry Portugal at 25°C	**1.**	5.81	4.46
	2.	5.58	4.55
	3.	5.62	4.73
Frankovka modra at 4°C	**1.**	5.24	4.40
	2.	4.85	4.35
	3.	5.18	4.29
Frankovka modra at 25°C	**1.**	5.40	4.56
	2.	5.63	4.22
	3.	5.20	4.61

TCB-total count of bacteria, A-*Acetobacte*r cells

Rapid and sensitive detection and enumeration of microorganisms has always been a challenge for the food industry **(Luo et al., 2004)**.

In the world of enology, AAB have received less attention than the other microorganisms involved in wine processes, but their activity during post fermentative operations and wine storage is quite a common problem in wineries. In the present study, rt-PCR and nested PCR, which have already been used with other bacterial groups **(Lyons et al., 2000; Luo et al., 2004; Rousselon et al., 2004)** were used, respectively, to quantify and detect AAB. We designed a specific set of primers for real-time amplification, but no positive results were obtained when they were tested against the usual wine microorganisms such as lactic acid bacteria and yeasts. Standard curves were obtained with DNA from several AAB strains, and the results were similar in all cases. Thus, any strain could be used for generating a standard curve for Acetic acid bacteria **(Lyons et al., 2000)**.

Acetobacter are more often isolated from wine, whereas *Gluconobacter* species are isolated from grape must. *Gluconobacter oxydans* is the main species found in association with grapes and grape must. *Acetobacter hansenii* and *Gluconobacter liquefaciens* have recently been reassigned as *Gluconacetobacter hansenii* and *Gluconacetobacter liquefaciens* **(Yamada et al., 1998)** and can be infrequently isolated in winemaking. The two species of *Acetobacter* most often isolated from wine are *A. aceti* and *A. pasteurianus* **(Silva et al., 2006)**.

The detection limit for the total AAB population was 10^3 cell/ mL, the same as for the individual species of *A. aceti, Ga. liquefaciens, Ga. hansenii*, and *G. oxydans*, while that of *A. pasteurianus* was 10^2 cell/ mL. The presence and sensitivity of Gram$^{-negative}$ bacterial species *Acetobacter aceti, Acetobacter pasteurianus* from the *Acetobacter genus, Gluconobacter oxydans, Gluconacetobacter hansenii* and *Gluconacetobacter liquefaciens* was detected in red wines using Real-Time PCR. Susceptibility of two *Acetobacter* species *A. aceti* and *A. pasteurianus* varied in different isolates from 10^2 to 10^5 CFU.mL^{-1}, the sensitivity of the species *G. oxydans* in different isolates of the wine samples ranged from 10^3 to 10^5 CFU.mL^{-1}. We also monitored in the two *Gluconacetobacter* species, which captured RTQ PCR sensitivity ranging from 10^3 to 10^5 CFU.mL^{-1}.

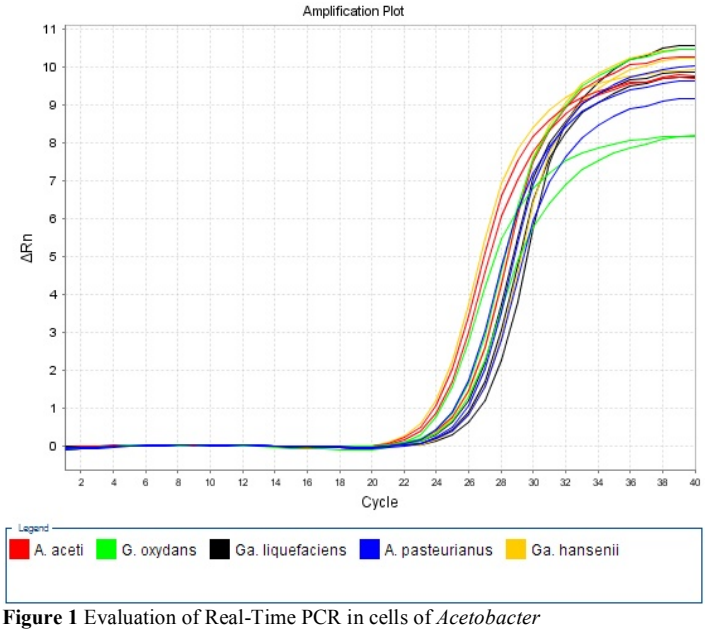

Figure 1 Evaluation of Real-Time PCR in cells of *Acetobacter*

The sensitivity, or detection limit, of our RT-PCR was 10^3 cells/mL but, in some species, values even reached 10^2 cells/mL. This detection limit, which is similar to those of other studies conducted with AAB **(Gonzalez et al., 2006; Andorrá et al., 2008)**.

The highest AAB species enumeration by RT-PCR was observed for *Ga. hansenii* and *Ga. liquefaciens*. These species were present in most of the samples, as well as *G. oxydans*. This latter species has been reported as one of the most commonly recovered AAB species in grape must, and its presence decreased during the fermentation, practically disappearing at the end of the fermentation **(Joyeux et al., 1984)**.

Ga. hansenii was another species that has been previously reported in wines in mid fermentation **(González et al., 2005)** and also at the end of fermentation **(Du Toit and Lambrechts, 2002)**. Curiously, *Ga. europaeus* was commonly found in vinegar **(Sievers et al., 1992; Trček et al., 2000; Vegas et al., 2010)** but not in wines. Another species frequently identified in wines at the end of fermentation was *A. pasteurianus* **(Du Toit and Lambrechts, 2002; Bartowsky et al., 2003)**, which was also detected in some of the wine samples; the recovery of this species on plate was possible from a single sample, despite its low abundance. *A. aceti*, which was often detected in wines from must to the end of fermentation, was poorly quantified in these samples **(Valera et al., 2011)**.

G. oxydans, *A. aceti*, and *A. pasteurianus*, are the ones that are most frequently found in the course of winemaking, as well as, to a lesser extent, *Gluconoacetobacter liquefaciens* and *Gluconoacetobacter hansenii*. The *G. oxydans* present on the grape disappears and gives way to *Acetobacter*, which subsists in wine **(Lafon-Lafourcade and Joyeux, 1981)**.

Gluconacetobacter liquefaciens in wine usually present towards the end of the alcoholic fermentation. This species acts as a spoilage organism, turning the ethanol resulting from the fermentation into acetic acid **(Sievers and Swings, 2005)**.

CONCLUSION

Although red wines are normally bottled with low oxygen content, but not always sterile filtered and the small resistant bacterial population is stay in wine. Especially red wine contains more than 10^3 CFU/mL^{-1}. Bottled red wines, sealed with natural cork closures, and stored in a vertical upright position may develop spoilage by acetic acid bacteria. This spoilage is evident as a distinct deposit of bacterial biofilm in the neck of the bottle. The oxygen content of this entrapped gas, which is finite, and minimized by the modern bottling equipment used, does not explain the often random nature of the acetic acid spoilage.

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