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Proceeding Paper

A Survey on Acetic Acid Bacteria Levels and Volatile Acidity in 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 through traditional microbiological methods, it would be advantageous to detect them using 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 via PCR using 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 that were wine-negative but must-positive for AAB had volatile acidity close to the acceptable limit. This study shows the utility of PCR diagnostics for predicting the risks of wine spoilage by AAB.

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

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1. Introduction

Acetic acid bacteria (AAB) are very widespread spoilage microorganisms in winemaking, and they 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 levels of sugars and ethanol [2], and they have high requirements for the presence of oxygen. When these AAB are present during winemaking, wine aging or wine storage, they metabolize ethanol to acetaldehyde using alcohol dehydrogenase and then produce acetic acid using acetaldehyde dehydrogenase [3], produce acetoin from lactic acid and ethyl acetate, and metabolize glycerol to dihydroxyacetone [4]. Moreover, they seem to affect wine quality by influencing must composition and alter the growth of yeast and lactic acid bacteria during fermentation [5].

One 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 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 its anamorph *Dekkera*, *Pichia anomala*, *Kloeckera apiculata* and *Candida krusei*) lead to the 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 and pre-bottling [11].

The European regulation (CE 1308/2013) has set out limits for sale at 1.20 and 1.08 g/L of acetic acid for red wines and white/rose wines, respectively [3], as has the legislation of the Republic of Moldova. These limits are provided by the regulation regarding the organization of the wine market in the 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 production. Primary strategies that could be mentioned are compliance with hygiene rules and regulations at wineries, the monitoring of nutrients and residual sugars during the fermentation and at the end of it, temperature control, the 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 wines.

Detection and quantification methods of the harmful microorganisms in winemaking are essential to preventing 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 difficult to cultivate using laboratory media, which highlights the importance of devising alternative methods for the detection of these bacteria [7]. Also, traditional methods require trained personnel, and final identification is performed through biochemical, physiological and morphology analysis via a microscopic examination, increasing the overall cost and limiting the test to the laboratory 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, i.e., 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 winemaking (grape, must, wine). Generally, direct methods have two major advantages over the indirect methods. Firstly, they can identify non-culturable microbes (those injured, viable but non-culturable or unable to grow using the chosen media). Secondly, the direct methods are much faster than indirect methods, since some microorganisms may require up to two weeks to grow [14]. In winemaking, the 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 a priority. Molecular biology methods, such as quantative PCR (qPCR), demonstrate high efficiency in the early detection and quantification of AAB and can be widely used in the winemaking process [15–17]. 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 [18]. It can also quantify PCR products with greater reproducibility while eliminating the need for post-PCR processing, thus preventing carryover contamination.

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