

Evaluation of the microbiome on untreated grapes and from the must obtained in Romanian rural households

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Abstract

Grapes possess a complex microbiome that includes yeasts, bacteria, and molds. This microbial diversity plays a significant role in the quality, flavor, and characteristics of traditional grape-derived products, making microbial monitoring crucial.

The microbial analysis of these microbial groups was carried out on two varieties of Romanian grapes, white and black, which are increasingly rare in Romania. Microbial groups, yeasts, bacteria, filamentous fungi and coliform bacteria were isolated from diluted suspensions obtained from the surface of white and black grapes and from the must squeezed from the two types of grapes. For the isolation of groups of microorganisms, specific nutrient media were used, such as nutrient agar for bacteria, agarized malt for yeasts, Sabourand for filamentous fungi, respectively Lauryl sulfate for coliforms. After incubation in appropriate conditions, it was observed that yeasts have the highest concentration, followed by bacteria. The smallest number was represented by filamentous fungi and coliform bacteria. Also, the number of Gram-positive microorganisms dominates. The load of yeasts, bacteria and filamentous fungi varies quantitatively depending on the changes in abiotic factors.

Keywords: yeasts, bacteria, filamentous fungi, must, coliforms

Introduction

Grapes are among the most well-known fruits at national and international level. Grapes make up 16% of global fruit production, with an estimated annual yield of around 68.9 million tons. The growing trend toward healthy eating has led to a significant increase in the demand for fresh grapes, with global consumption reaching 24.1 million tons [23]. In Romania, the production of grapes was approximately 736,892 tons in 2016, and the average consumption of grapes increased by 4.17% on average compared to 2010. In the same year, the average consumption of grapes per capita was of 6.4153 kilograms of grapes [12]. Some studies predict that by 2025, the average annual grape consumption per person will exceed 23 kg [31]. Grapes offer numerous benefits for human health, due to the richness in bioactive compounds (phenolic acids, flavonoids, anthocyanins), fibers, natural sugars, lipids, mineral substances and vitamins [11].

Due to the complex phytochemistry, grapes have antimicrobial, antitumor, anti-inflammatory and high antioxidant potential [14, 15, 23]. Eating grapes significantly enhances the immune system, providing essential nutrients that strengthen the body's defenses [13]. In addition to these health benefits, grapes reduce cardiovascular risks and have wide uses in the food, pharmaceutical and cosmetic industries [29]. Grapes represent a natural reservoir of microbial species that can influence the quality of grapes, must and the winemaking process. Microorganisms on different organs of plants, including on fruits, have attracted the attention of the scientific world due to their applicative potential in the field of biotechnologies. In addition, rising concerns about antimicrobial resistance, restrictions on chemical treatments for disease control, and consumer preferences for healthy, flavorful, locally-grown Romanian fruits have intensified the study of microorganisms.

This has led to a growing search for safer solutions to protect grapes from pathogens and to enhance the natural protective microbial barrier.

Information about the composition of grapes, fermentation yeasts, winemaking processes is numerous [24], but studies oriented towards biodiversity and the importance of epiphyte microflora are less. This, along with the previously mentioned factors and the population's shift towards healthier eating habits, prompted us to evaluate the presence of bacteria, fungi, and coliforms on the berries of various Romanian grape varieties. Research indicates that the microflora found on grapes originates from their surrounding environment [4, 32]. Compant et al. (2010) [5], mention that soil microorganisms populate the aerial part of plants. The indigenous microorganisms on grapes are a natural barrier against pathogens and can quantitatively and qualitatively influence the harvest and the products obtained from grapes [24]. The presence of pathogenic microorganisms not only degrades the quality of grapes, affecting their taste, texture, and overall market value, but also poses significant health risks to consumers, such as foodborne illnesses and infections. For these reasons, we conducted a thorough analysis of coliforms, which are key indicators of contamination and potential disease-causing pathogens. The microbiome on grapes undergoes continuous changes during ripening, varying both in quantity and composition. This effect is mainly due to anthropogenic (chemical treatments) and abiotic factors (temperature, light, humidity, soil properties) [2, 6, 18]. The evaluation of the phyllosphere of the vine, flowers and grape berry surfaces, showed that the microbial communities are mainly composed of representatives of *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria* [27].

Material and Method

Collection of samples to be analyzed

The samples of white and black grapes originated from two gardens in Caras-Severin county, namely Black Frankincense and White Frankincense varieties. The stumps are planted in the garden and the ropes are traditionally stretched over the fence. Soil enrichment and protection of the vine at ground level was carried out with cattle manure in case of white grapes and sheep in case of black grapes. The grape samples were harvested in autumn and transported under appropriate conditions to the microbiological analysis laboratory of the University of Life Sciences "King Mihai I" from Timisoara.

Microbiological analysis of grape and must samples

20 g of each grape sample was used to obtain the must and evaluate the microflora on the surface of the grape seeds. For the study of the surface microbiome, the grape seeds were washed with physiological serum. For the study of the microbiome in the pulp of grape seeds, a must was obtained from the surface and seeds by crushing the grape samples. The microbial suspensions on the surface of the grapes and the must were diluted with physiological serum, by the dilution method. To enumerate the bacteria from the microbial suspensions on the surface of the grapes and in the must, the 10^{-6} dilution (1 ml/10 ml medium) was inoculated on Agar Plate Count (PCA) [4, 14, 20]. The fungi were isolated from the 10^{-3} dilution on Sabouraud nutrient medium. Yeasts were grown on medium with malt. The isolation was carried out in two repetitions. The favorable temperature for growth was established at 28°C. The protection of the vines and the enrichment of the soil in their area was carried out with manure, which can be a source of contamination with coliform bacteria. After isolating the microbial cultures, macroscopic and microscopic studies were carried out to establish the dominant bacterial and fungal species. It is known that edaphic microorganisms reach different parts of the plant. This was a reason why a number of coliform bacteria was also established. Coliform bacteria were isolated on Lauryl-Sulfate medium in three repetitions. The first three dilutions were used for inoculation. The test tubes also contained Durham tubes for capturing the gases produced as a result of the fermentation processes. After incubation at 37°C, a probable number of coliforms was established [21, 25]. Triple sugar iron (TSI), Sim and Citrate Simmons (CS) media were used to confirm the presence of coliforms. The bacteria were incubated for 48 hours, the coliforms for 24 hours and the fungi for 5-7 days [20].

Results and Discussion

Grapes' microbial ecology is complex, consisting of filamentous fungi and bacteria that have physiological peculiarities and different effects on fruits and wine products. Some species are found only in grapes, such as parasitic fungi and environmental bacteria, while others have the ability to survive and grow in wine products, constituting their microbial consortium. This microbial consortium includes species of yeasts, lactic and acetic bacteria. The obtained results show that the microbiome of intact grains is dominated by yeasts ($0.96-4.50 \times 10^2/\text{mL}$), followed by bacteria ($0-0.84 \times 10^2/\text{mL}$) and molds ($0-0.37/\text{mL}$) (Figure 1). The samples were analyzed after ripening and harvesting, when, according to some studies, yeasts dominate the microbial consortium, and the proportion depends on the degree of ripening of the grapes and the availability of nutrients.

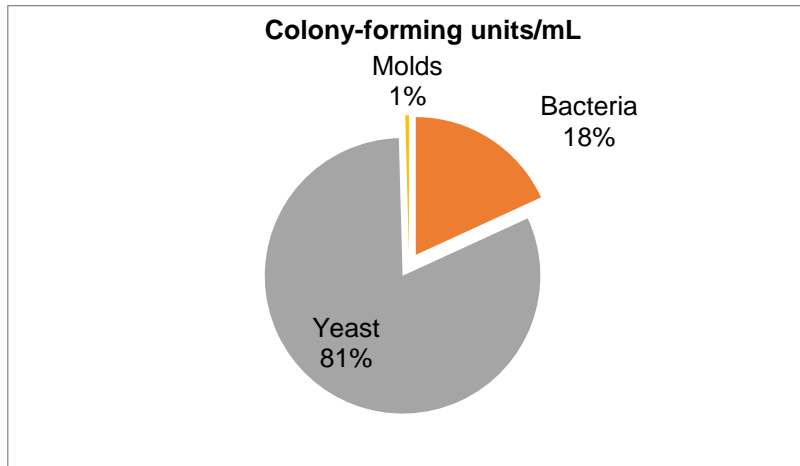


Figure 1. The proportion of microorganisms on the surface of the berries and in the must of white and black grapes

Quantitatively, the microorganisms on the grape berries reach values between 10^4 – 10^6 CFU/g [9]. In our studies, microorganisms have lower values. Bacteria were isolated in large numbers from black grapes, but were absent from white grapes. Bacteria were highlighted in both types of must. The descending order of the concentration of bacteria in the four samples was BG2 > GM1 > GM2 (Figure 2). Non-pigmented "S type" bacterial colonies dominated in all samples (Table 1). Numerous studies demonstrate that the abundance or reduction of microbial communities on grapes is influenced by the variety and the management applied [27], the origin of the vineyard [10], the geographical area [7], the grape development phase [18, 30] meteorological conditions, but it can also vary from one bunch to another [1, 30]. It has been proven by some authors that grapes are contaminated with bacterial species from the *Bacillales*, *Enterobacteria*, *Lactobacillales*, and *Pseudomonadales* orders [7]. We observed species from the genus *Bacillus* and *Micrococcus* in the samples we analyzed, in a manner similar to these results. Also, both Gram positive and Gram negative species, bacillary forms and cocci were highlighted. Some investigations have shown that species from the genera *Bacillus sp.* and *Pseudomonas* can play a major role in biological control, protecting plants and stimulating their growth [5, 31].

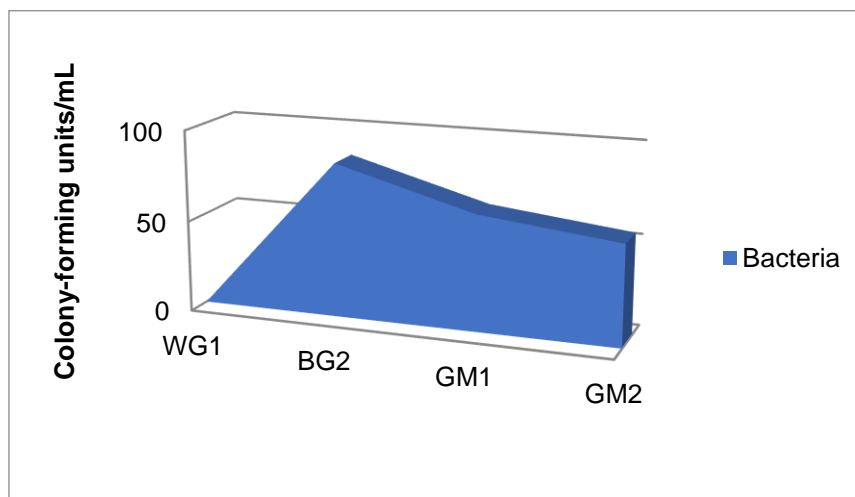


Figure 2. Estimation of bacteria from the analyzed samples
Legend: WG1-white grapes; BG1-black grapes; GM1- white grape must; GM2- black grapes

Table 1. Characteristics of colonies grown on nutrient media

Samples	Colony characteristics	Caracter Gram
WG1-white grapes	-	-
BG2-black grapes	regular, non-pigmented, small, convex, glossy, smooth colonies dominate; 4 large, irregular, translucent, matte, non-pigmented, umbonate colonies	bacillary forms and cocci Gram positive and Gram negative
GM1- white grape must	regular, non-pigmented, small, convex, glossy, smooth colonies	Most of them are cocci, arranged in piles Gram positive
GM2- black grapes must	regular, non-pigmented, small, convex, glossy, smooth colonies dominate; Out of the total, 36 are large, flat, translucent colonies characterized by a wrinkled surface.	Cocci and bacillary forms Gram positive

It has been proven by most studies that yeasts are the most common microorganisms on grapes. Along with these, groups of lactic and acetic bacteria were found in particular [9]. In the samples analyzed by us, the yeasts were in greater numbers in the must, compared to the bacteria. The highest number of yeasts was observed in the must of white grapes, followed by black grapes. The lowest number of yeasts was highlighted on white grapes (Figure 3).

Grapes are susceptible to contamination by various molds, and fungal diseases can affect their quality and chemical composition, as well as the aroma and color of must and wine. Some molds are responsible for grape rot and produce toxins [18]. Knowing these microorganisms is essential, because [26], mention that some of these microorganisms could be used in the protection of plants and in the control of phytopathogenic agents. According to Wei et al. (2022) [30], the grapes' development stage influenced the diversity of microorganisms (bacteria and fungi). In our analyses, molds were present in higher numbers on white grapes, followed by the must obtained from this variety. The number of molds was reduced on black grapes. Molds did not develop in the black grape must (Figure 4). So, in the samples where bacteria dominated, the number of fungi was reduced, and vice versa. Among the fungi, the genera with septate hyphae, *Aspergillus* and *Penicillium* were especially present. In accordance with our results are those obtained by other authors [19]. In contrast, Felšćiová et al. (2023) [8] observed in the analyzed grapes that the genera *Alternaria* and *Botrytis* are the most abundant.

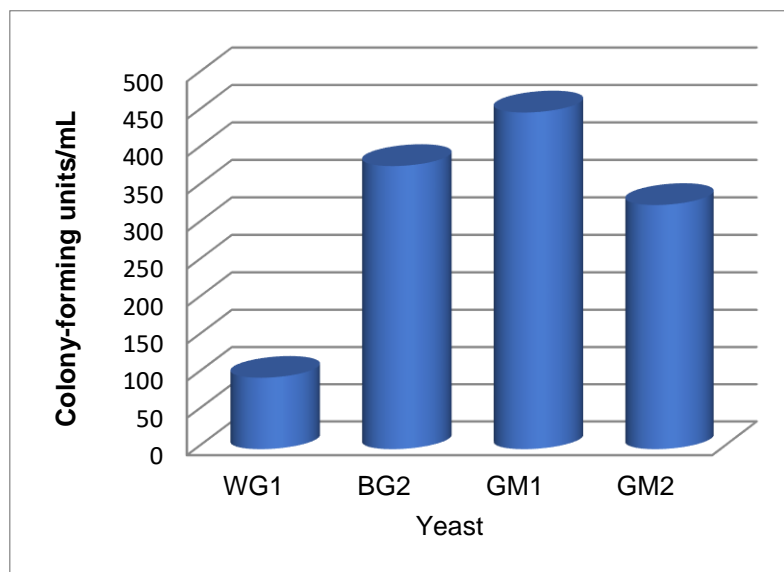


Figure 3 Quantitative evaluation of yeasts from the analyzed samples
Legend: WG1-white grapes; BG1-black grapes; GM1- white grape must; GM2- black grapes

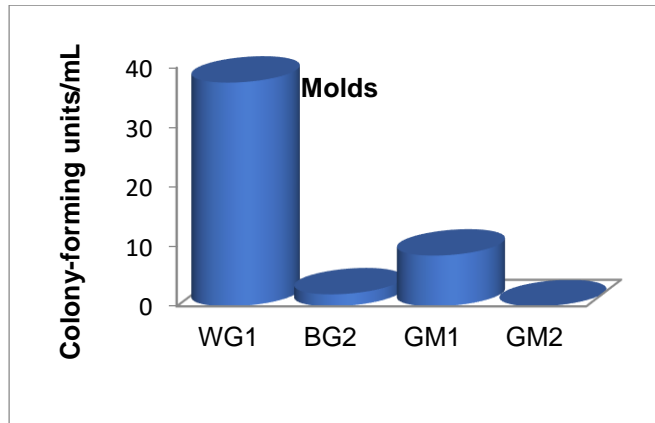


Figure 4 Quantitative evaluation of molds from the analyzed samples
Legend: WG1-white grapes; BG1-black grapes; GM1- white grape must; GM2- black grapes must

The abundance of coliform bacteria is generally affected by the differences in the composition of the analyzed samples, storage, sale, and processing conditions [24]. Microorganisms on grapes also have telluric origins. Manure can be a source of coliforms, and our experiments revealed the isolation of coliform bacteria in fairly low quantities, with the must samples showing the highest count among the four samples tested (Figure 5). *Proteus* and *Klebsiella* species were identified on the confirmation media. The presence of coliform bacteria and the confirmation of bacterial species from the *Enterobacteriaceae* family on fruits, including grapes, is also demonstrated by other authors [3, 28].

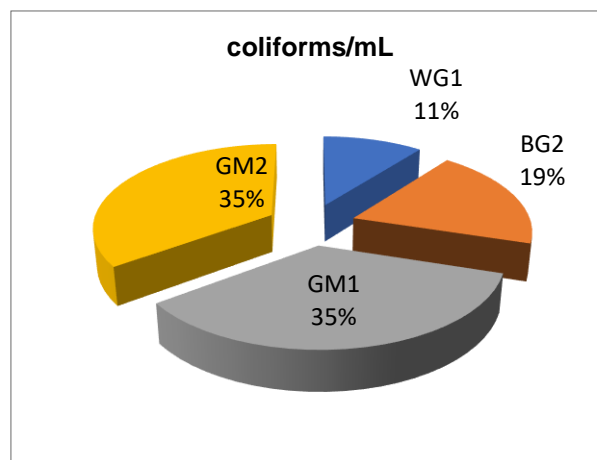


Figure 5 Probable number of coliform bacteria in the evaluated samples
Legend: WG1-white grapes; BG1-black grapes; GM1- white grape must; GM2- black grapes must

Therefore, microorganisms are found on the surface of the grapes as well as in the must obtained from crushed pulp and skin.

Conclusions

The presence of beneficial bacteria and yeasts on the grapes and in the must is advantageous for consumers, fermentation processes, and viticultural products. While the low levels of coliforms found are below the thresholds set by current regulations and do not pose significant issues, they underscore the importance of washing grapes before consumption. Fungi are few in number and include representatives of the genera *Aspergillus* and *Penicillium*. Some species of these genera are toxin producers and must be monitored if they are in high concentrations. This study offers insights into the variations in microbial communities on grape surfaces post-harvest. Future research will focus on examining changes in the microbial community within the

rhizospheric zone, on various vine organs, and at different stages of grape development to enhance our understanding of the microbiome's ecology on the fruit and to promote grape health.

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