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Microbial Diversity of Grape Varieties from Three Geographical Indication Regions of the Republic of Moldova

253.03 - TECHNOLOGY OF ALCOHOLIC AND NON-ALCOHOLIC BEVERAGES

Summary of the Doctoral Thesis in Engineering Sciences

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CONTENT

I. RESEARCH CONCEPT GUIDELINES

The actuality of the research and the importance of addressed issue: The International Organisation of Vine and Wine (OIV) defines "*terroi*r" as an area in which collective knowledge about the interactions between human and natural factors shapes the unique characteristics of the wine produced. Microbial diversity, also known as "*microbial terroir*" is an important component of a wine's *terroi*r. This concept was introduced into the field of oenology a few years ago, thanks to the development of next-generation sequencing (NGS) techniques that allow the identification of the microbial ecosystem in various wine regions using specific analytical methods.

Grape varieties and their geographical origin have always been considered the most important factors determining the microbial diversity of grapes. Within the concept of "*microbial terroir*" the microbial diversity of several indigenous varieties has been identified; for example, Barbera d'Asti DOC [1], California Zinfandel [2], as well as some international varieties such as the red Cabernet Sauvignon and the white Chardonnay, which are also popular in this context.

At the national level, the wine industry is an essential sector economically, strategically, socially, and culturally for the Republic of Moldova. It constitutes the most important part of the agricultural and food sector, accounting for over 16% of the total agricultural output. According to the National Bureau of Statistics of Moldova (BNS), Moldovan wine exports generated an income of 3.1 billion lei in 2019, representing approximately 12% of the external trade balance and 3% of the Gross Domestic Product (GDP).

Currently, vineyards occupy 7% of the total agricultural land in the Republic of Moldova and 3.8% of the country's total area, demonstrating the highest vineyard density in the world—estimated at 3.44 hectares per 100 inhabitants. The sector comprises an estimated more than 50,000 farmers and peasant households,

250 agricultural enterprises, 181 wineries, and 10 agricultural cooperatives. In this sector, more than 150,000 people are employed directly or indirectly, accounting for over 15% of the country's active workforce.

In this context, recent research in wine microbiology has begun to focus on the isolation and use of local yeast strains/species in the production of natural, organic, and sustainable wines that exhibit the organoleptic characteristics typical of the vineyards where the grapes are grown. For example, studies conducted by Taran, N. et al. on the indigenous Codrinschii variety led to the selection of nine yeast strains with high biotechnological potential for the production of dry red wines [3]. These local yeast strains are capable of adapting to specific environmental conditions, fermenting the sugars in the must, and contributing to the production of wines with typicity and high organoleptic qualities.

The study by Wang et al. contributes to the understanding of Moldovan "*microbial terroir*" by exploring the Fetească Neagră wine from the Republic of Moldova. The research results demonstrated notable differences between micro- and macro-terroir, highlighting the influence of local factors on the microbial composition and on the unique characteristics of the wine [4].

In recent decades, wine production has been based on commercial yeast strains of *Saccharomyces cerevisiae* as starter cultures, and the role of non-*Saccharomyces* yeasts has been largely ignored. Recently, researchers Roudil L. and Russo P. discovered that non-*Saccharomyces* yeasts can positively contribute to the aroma, quality, and food safety of wine. Non-*Saccharomyces* species such as *Torulaspora delbrueckii*,*Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Schizosaccharomyces pombe*, and *Pichia kluyveri* are already available on the market as commercial cultures suitable for various winemaking needs [5].

At the same time, bacteria have not been studied as extensively as fungi, with interest mainly focusing on lactic acid bacteria (LAB) and acetic acid bacteria (AAB). LAB include

genera such as *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, *Enterococcus*, *Weisell*a, and *Oenococcus oeni*, which are frequently used in winemaking. AAB include genera such as *Acetobacter* and *Gluconobacter*, which have been identified in various studies [6]. In a study conducted by Zgardan D. et al. on Moldovan wines, the presence of AAB particularly the species *Acetobacter pasteurianus* was reported at all stages of winemaking, leading to an increase in volatile acidity beyond acceptable limits [7].

It is well known that grape must fermentation and wine production are complex processes that involve the metabolic activities of various types of microorganisms. During alcoholic fermentation, most of the sugar content is metabolized by yeasts to produce ethanol and carbon dioxide, making yeasts the key players in this technological process. However, recent findings by Roudil L. suggest that the role of non-*Saccharomyces* yeasts and certain bacterial species in alcoholic fermentation has been underestimated, and interest in both non-*Saccharomyces* yeasts as potential starter cultures and bacterial diversity is growing, given that they contribute to the sensory properties of wine through the production of various metabolites [5].

For a long time, a microorganism was considered viable only if it was cultivable—that is, if it could be grown under optimal conditions to form an inoculum in liquid media or colonies on nutrient agar-based media. However, there exist microorganisms that are unable to form colonies on nutrient media, yet their cells remain biochemically active; these are referred to as viable but non-culturable (VBNC) microorganisms.

The surface of the grape berry is an unstable habitat that changes according to climatic conditions, which in turn depend on several natural factors such as temperature, humidity, and UV radiation [7]. Yao determined that the Cool Night Index (the index of nighttime temperatures during grape ripening) has the greatest effect on the yeast population on the berry surface [8], while

Combina et al. confirmed that precipitation close to the time of grape harvest affects the quantity and quality of yeasts on the grape berry surface [9].

Although numerous studies have been conducted so far, the available results do not allow for a deep understanding of the influence of climatic factors on the microbial ecosystem characteristic of the wine regions in Moldova.

Another significant impact comes from human factors such as agricultural practices, the use of fertilizers, irrigation, weed management, and the application of plant protection products (pesticides, insecticides, and herbicides), all of which fundamentally influence both the qualitative and quantitative aspects of the grape yeast/microbial ecosystem. The use of plant protection products causes quality and food safety issues, such as excessive residues in wine, changes in its organoleptic characteristics, and toxic effects on consumers. Etienne et al. found that fungicide treatments affect the yeast community on the grape berry surface [10].

The use of microbial resources in winemaking represents a significant factor in innovation and quality improvement within the wine industry. The current exploration of a wide range of species both *Saccharomyces cerevisiae* and non-*Saccharomyces* to improve wine characteristics and to meet the continuously evolving preferences of consumers is generating a competitive and sustainable wine industry [11].

Native yeast must (MLI) is a traditional winemaking technique that uses a small amount of partially fermented must as an inoculum to initiate a new fermentation in another volume of must. This method aims to ensure the stability and efficiency of the fermentation process through the rapid proliferation of the microbial population at the initial stages. MLI can be derived either from laboratory-selected yeast cultures or from musts undergoing spontaneous fermentation, and the characteristics of the microbiome at this stage directly influence the aroma and final

character of the produced wine. For example, the use of laboratory-selected *Saccharomyces cerevisiae* strains can guarantee a constant fermentation rate and more precise control of the process. On the other hand, employing an MLI derived from spontaneous fermentations can preserve the authenticity of the terroir's microorganisms, conferring the wine with a unique aromatic complexity and distinct regional expression. However, the successful application of MLI requires rigorous control of microbial diversity and fermentation conditions to prevent the proliferation of wild yeasts or other unwanted microorganisms that could affect the final product's quality.

Bioprotection represents an emerging technology in winemaking, aimed at inhibiting the development of harmful microorganisms by introducing non-*Saccharomyces* yeasts or other beneficial microorganisms, thereby reducing the need for sulfur dioxide (SO_2). Recent studies by Rubio-Breton P. et al. have shown that yeasts such as *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, and *Lachancea thermotolerans* can limit the growth of unwanted microorganisms in the early stages of alcoholic fermentation through mechanisms of competitive inhibition and the secretion of antimicrobial substances. These yeasts not only ensure the microbiological stability of the wine but also contribute to its aromatic complexity by producing unique metabolites (aromatic esters and organic acids). Additionally, bioprotection technology helps reduce the amount of SO_2 used, thereby meeting consumer demands for more natural, eco-friendly, and organic wines. However, similar to MLI, the effectiveness of bioprotection depends on the appropriate selection of strains, the inoculation dose, and precise management of fermentation conditions.

The purpose and objectives of the research is to investigate the microbial diversity associated with grapes produced in the three geographical indication (GI) zones in the Republic of Moldova and to apply commercial and/or indigenous microorganisms in the production process of typical wines with

minimal intervention, particularly in organic wines.

To achieve this aim, the following **specific objectives** have been set:

- quantify the microorganisms present on grapes produced in the three GI zones of Moldova;
- study the factors influencing the microbial diversity of grapes grown in the three GI zones;
- isolate and multiply the selected microorganisms on different culture media;
- utilize amplicon-based next-generation sequencing (NGS) technology for the qualitative analysis of the microbial community associated with grapes grown in the three GI zones;
- develop an innovative wine production strategy by applying commercial and/or indigenous microorganisms in the production of batches of organic wines.

Research hypotheses:

1. The quantity of yeasts on the grape berry surface and in the must is influenced by climate (the natural factor) and by vineyard management practices (the human factor).
2. The Republic of Moldova possesses a unique grape microbiome structure with differential variations in the three GI zones and among the grape varieties cultivated in these vineyards.
3. The particularities of microbial diversity can be applied to defining and developing the typicity and authenticity of wine products from the vineyards of the Republic of Moldova.

II. MATERIALS AND METHODS

The study was carried out within the "Quality Grapes" project of the National Office of Vine and Wine (ONVV) at the SARCO/EXCELL laboratory (France). Research on microbial quality was conducted at the company Tinygene, Ltd. (Shanghai, China). The validation of the research results and the implementation of the technology for valorizing the microbial community in the production of organic wines were carried out at ÎM "Vinăria Purcari" S.R.L. during the author's internship.

Additionally, the author of this study was supported by the Chinese Council through a Study Scholarship. As a result of implementing the innovative technology based on the research outcomes, the "Native de Purcari" wine was produced. The investigation of the organoleptic characteristics, as well as the dynamics of the physicochemical and organoleptic parameters, was carried out in specialized laboratories at the Moldo-French company LACO-ALFATEC S.R.L., the "Verification of Alcoholic Production Quality" unit of INCAAMV, the Center for Oenological Research of the Faculty of Food Technology/UTM, and the Institute for Research on Special Plants and Animals of the Chinese Academy of Agricultural Sciences.

2.1 General Characteristics of the Methodology for the Microbiological Research of the Indigenous Grape Flora

For the microbiological study, 38 grape samples were selected, each having a specific importance within the analysis. These samples were collected from various locations over three consecutive harvest years (2018–2020), thereby providing as complete a picture as possible of the variability of the microbiome associated with the vine. The study samples were grouped based on three main criteria: grape variety, harvest year, and the GI region from which they were collected. This classification allows for a detailed analysis of how different factors influence the diversity and composition of the grape-associated microbiome.

For NGS sequencing, 9 samples (CCS, CCH, CFN, SCS, SCH, SFN, VCS, VCH, VFN) were selected from three grape varieties across the three GI regions. These samples enabled the acquisition of a detailed picture of the microbial diversity at the molecular level and the identification of microbial specificities that can influence terroir characteristics.

The methods for quantifying microorganisms were epifluorescence microscopy (EFM) and quantitative analysis on specific culture media (MCS). With the EFM method, viable microorganisms are counted using a protocol and materials

developed by Chemunex. Samples from the grape washing water and must are filtered through a Chemfilter CB04 membrane, which is then incubated at 30°C for 30 minutes in darkness. The initially non-fluorescent substrate is cleaved by a cellular enzymatic system that releases a fluorochrome. This fluorochrome, when excited by ultraviolet light at 480 nm, emits green fluorescence. The Chemfilter membrane, placed between the slide and the cover slip of the epifluorescence microscope (Olympus BX51) equipped with the appropriate filter (Olympus 467803), is observed at a magnification of 1000.

For the quantitative analysis (MCS) used to count yeasts, samples from the grape washing water and must are plated on Petri dishes containing 10 g/dm³ yeast extract, 10 g/dm³ bacterypton, 20 g/dm³ glucose, 25 g/dm³ agar, and 0.015% biphenyl. For LAB bacteria, the medium contains 500 ml/dm³ grape juice, 5 g/dm³ yeast extract, 2 ml/dm³ L Tween 80, and 20 g/dm³ agar. For the cultivation of AAB bacteria, a similar medium was used, supplemented with 30 mg/dm³ penicillin.

2.2 The Experimental Protocol Used in the Production of Wines under Bioprotection and in the Preparation of MLI

During the period 2021–2023, oenological practices were carried out focusing on the bioprotection process and the development of wines with MDI. The aim of this research included the use of non-*Saccharomyces* yeasts as bioprotection agents in wine production to reduce the use of sulfur dioxide, as well as the use of MLI instead of commercial yeasts to explore and valorize the Moldovan terroir.

The study samples were of the Rară Neagră variety, sourced from an organic vineyard in Olănești, Ștefan Vodă, Republic of Moldova. The grapes were hand-harvested at full technological maturity (total sugar content of 255 g/dm³, titratable acidity of 4.65 g/dm³ as tartaric acid) and under perfect hygienic conditions. The grape processing followed the classic method, from which two study batches were produced: two tanks of 160 dal as the control

sample (group C1) and two tanks with bioprotection (group BP). In the control group C1, 100 mg/dm³ of potassium metabisulfite (PMS) and 12 mg/dm³ of pectolytic enzyme (LAFFORT LAFAZYM) were added. In the BP group, two types of non-*Saccharomyces* yeasts (*Torulaspora delbrueckii* and *Metschnikowia pulcherrima*) along with pectolytic enzymes were added. During alcoholic fermentation, both punching down and pumping over were performed twice a day, and the density was measured twice (in the morning and in the afternoon). In the final phase of fermentation, at a density of approximately 1010–1020 kg/m³, 25 mg/dm³ of LAFFORT LACTOENOS was added. When the sugar content dropped below 4 mg/dm³, hydraulic pressing of the fermented pomace was carried out. The experimental wines, after decanting from the yeast sediment, were transferred into 22.5-dal barrels for maturation over 6 months. Before bottling, the wines were sulfited with 50 mg/dm³ of PMS.

For the preparation of traditional MLI must, the grapes were pressed without decantation; the obtained must was sulfited with 20–30 mg/dm³ SO_2 and maintained at 25 °C until spontaneous fermentation commenced. For the module using traditional MLI, grapes of the Pinot Noir variety were processed according to the procedure described above. The contents were grouped into two study batches: two tanks of 160 dal as the control sample (group C2) and two tanks with MLI (group MLI). In both the control group C2 and the MLI group, 100 mg/dm³ PMS and 12 mg/dm³ of pectolytic enzyme (LAFFORT LAFAZYM™ CL, France) were added. After must clarification, commercial yeast (LAFFORT ZYMAFLORE™ 011 BIO, France) at 80 mg/dm³ was inoculated in the control group, while in the MLI group MLI was inoculated at 3% of the volume. Subsequently, the technological procedures were similar to those described for groups C1 and BP.

In the study with optimized MLI, the local variety Fetească Neagră was used to produce wines that better reflect local characteristics. The experimental vinification consisted of three

technological stages: (1) Preparation of the base medium for MLI-T (traditional MLI) / MLI-O (optimized MLI) by adding wine, so that the final ethanol concentration in the must is 1.5% vol. (MLI-O) or water (MLI-T) to the must; (2) Spontaneous fermentation for 3 days to obtain MLI-O and MLI-T; (3) Inoculation of MLI-O, MLI-T, and commercial yeast (control group) into fresh must. All technological schemes were carried out in triplicate using the same batch of grapes (three tanks for each trial), with the commercial strain of *S. cerevisiae* (LAFFORT ZYMAFLORE) serving as the control.

2.3 Analytical Methods for Studying the Quality and Composition of the Experimental Samples

Gas chromatography with flame ionization detection (GC-FID) for detecting the main volatile compounds; Capillary electrophoresis (CE) for the analysis of organic acids; High-performance liquid chromatography (HPLC) for the analysis of anthocyanins; and Fourier-transform infrared spectroscopy (FTIR) for analyzing physicochemical properties. In parallel with the quantitative analysis of the experimental wine samples, an organoleptic analysis was also performed. The wine tasting process took place in the specialized tasting room of ÎM "Vinăria Purcari" S.R.L. The tasting panel consisted of six experienced evaluators (2 women and 4 men, aged between 26 and 62 years).

2.4 Statistical Analysis of the Results

In the analysis of the quantitative content of the grape microbiome, during the alcoholic fermentation process, and throughout the storage of the produced wines, the following statistical tests were employed: post-hoc Tukey tests, analysis of variance (ANOVA), principal coordinate analysis (PCoA), non-parametric analysis of similarities (ANOSIM), and principal component analysis (PCA).

All statistical procedures were carried out using R Project for Statistical Computing, version R.4.04; for the Pearson

correlation of the NGS results, a significance level of $p < 0.01$ was applied, while for all other results, $p < 0.05$ was considered significant.

III. MICRORBIAL DIVERSITY IN THE GI REGIONS OF THE REPUBLIC OF MOLDOVA

Based on studies conducted on the characteristic microbiome of the three GI zones in Moldova focused on the microbiological analysis of 38 grape samples from the 2018–2020 harvests, 8 wine batches, and specific physicochemical analyses the following technological aspects with industrial application have been established.

3.1. Study of Microorganisms Using Culture Methods

The total number of yeasts on the surface of the grape berry (NLS-M1) ranges between 2.2E+04 and 3.9E+07 CFU/berry, while the number of culturable yeasts on the grape berry surface (NLS-M2) ranges between 4.1E+02 and 8.0E+06. The total number of yeasts in the must (NLM-M1) ranges from a very low level (<8.9E+02) to a level exceeding 3.1E+06 CFU/berry, whereas the number of culturable yeasts in the must (NLM-M2) ranges between 3.0E+03 and 2.0E+05. To compare the yeast counts among different varieties, regions, and harvests, boxplots were created (Figure 3.1.).

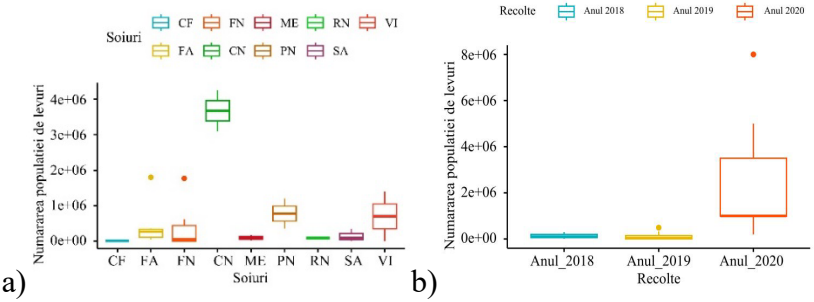


Figure 3.1. Box-plot of yeast counts grouped by different grape varieties and harvest years.

Based on the studies, it was determined that the total number

of yeasts in the must differs significantly among the grape varieties (P = 9.75e-05), especially in the case of the Couderc Noir (CN) variety from home gardens where no phytosanitary products were used. In addition, the number of culturable yeasts on the grape berry surface varies significantly between harvest years (P = 0.00251).

Through in vitro cultivation, several species of yeasts and bacteria were isolated, including *Rhodotorula glutinis*, *Metschnikowia pulcherrima*, *Aureobasidium pullulans*, *Hanseniaspora uvarum*, *Pediococcus damnosus*, *Lactobacillus brevis*, *Lactobacillus fermentum*, and *Gluconobacter oxydans*. The results indicate that the genetic profile of the *S. cerevisiae* strain isolated from the 2020 harvest is completely different from those obtained in 2018 and 2019; however, the two *S. cerevisiae* strains isolated from the 2019 harvest are identical.

To better assess the relevance of the results, the Pearson correlation coefficient was calculated for various factors and the yeast counts determined by different methods in different samples (Figure 3.2).

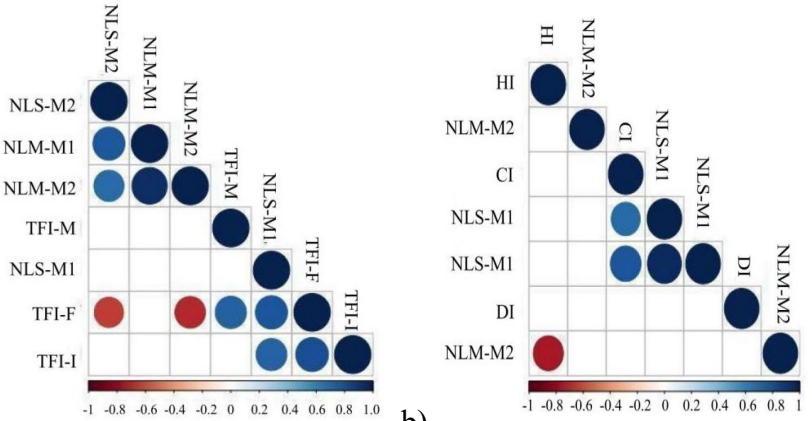


Figure 3.2. Pearson correlation: a) between the frequency index of phytosanitary treatments (TFI) and yeast counts, b) between climatic factors and yeast counts.

In Figure 3.2, the blue circle indicates a positive correlation,

the red circle indicates a negative correlation, and the open circle indicates insignificant results. The relationships between TFI_F (phytosanitary products for powdery mildew) and NLS_M2 (the number of culturable yeasts on the berry surface) and NLM_M2 (the number of culturable yeasts in must) are negative, but the relationship is positive with NLS_M1 (the total number of yeasts on the berry surface).

These findings offer a new perspective: phytosanitary products used to combat powdery mildew reduce the number of culturable yeasts, but increase the total number of yeasts on the berry surface. This is explained by the fact that sulfur-based phytosanitary products are effective in protecting the grapes against powdery mildew, with sulfur having a proven antimicrobial effect on yeasts, which likely causes the microorganisms to transition to a viable but non-culturable (VBNC) state.

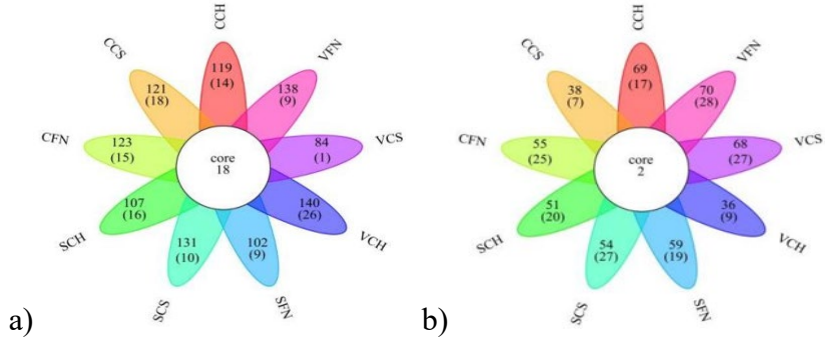
Regarding climatic factors, the HI (Huglin index) is negatively correlated with NLM_M1 (the total number of yeasts in must), meaning that higher temperatures during the growing season reduce the total yeast count in the grape must. Another temperature index, CI (the index of cold nights before harvest), is positively correlated with both NLS_M1 (the total number of yeasts on the berry surface) and NLS_M2 (the number of culturable yeasts on the berry surface). In other words, the cold temperatures during the nights before harvest inhibit both the culturable yeasts and the total yeast count. Climatic factors are very complex, and there are very few explanations for the variations in yeast numbers based on this factor.

As mentioned in the first chapter, current research on climatic factors focuses more on the impact on certain specific microbial populations.

3.2. Study of Microbial Diversity Using NGS Sequencing

To analyze the similarity and overlap of the microbial OTU composition, a Venn analysis of alpha diversity was performed

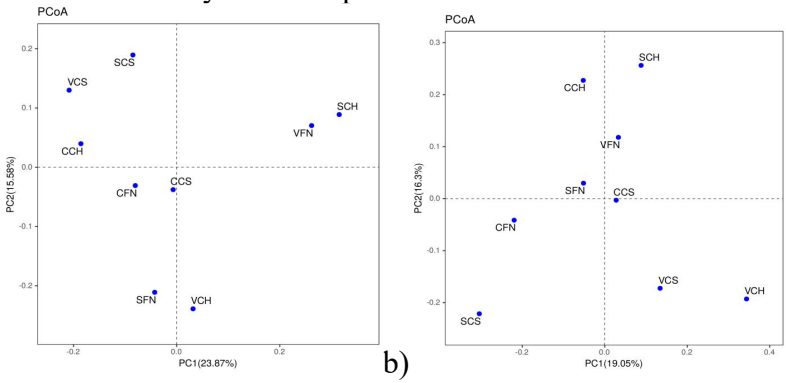
(Figure 3.3). In this figure, the outermost circle displays the sample name, while the petals include two lines of numbers: the upper line represents the total number of OTUs contained in each sample, and the lower line (in parentheses) indicates the number of unique OTUs for each sample; the white circle in the middle represents the core OTU count.



a) b)

Figure 3.3. Venn diagrams of the number of OTUs: a) for fungi, b) for bacteria.

According to the data presented in the Venn diagram, the IGP Valul lui Traian region emerged as the region with the richest microbial diversity in the Republic of Moldova.



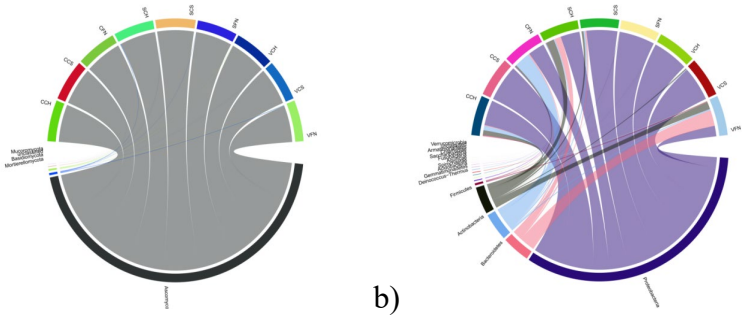
a) b)

Figure 3.4. Principal Coordinate Analysis (PCoA) Based on Unweighted UniFrac Distances for a) Fungi and b) Bacteria

Based on the PCoA analysis (Figure 3.4) of beta diversity,

no similarities were found in the structure and composition of the fungal and bacterial communities, either among varieties or between regions. This result may be due to the uncertainty inherent in spontaneous fermentation.

At the phylum level, the fungal microbial composition in all samples (Figure 3.5a) included representatives from 5 phyla: *Ascomycota* (98.8%), *Mortierellomycota* (0.6%), *Basidiomycota* (0.5%), *Mucoromycota* (0.06%), and *Rozellomycota* (0.04%). Only the VCH sample contains *Rozellomycota*.



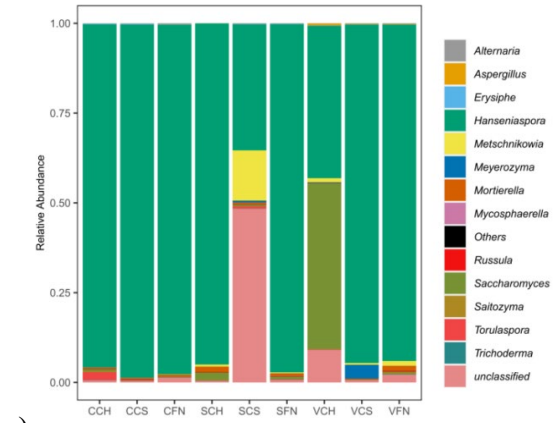
a) b)

Figure 3.5. Microbial Structure at the Phylum Level: a) for Fungi, b) for Bacteria

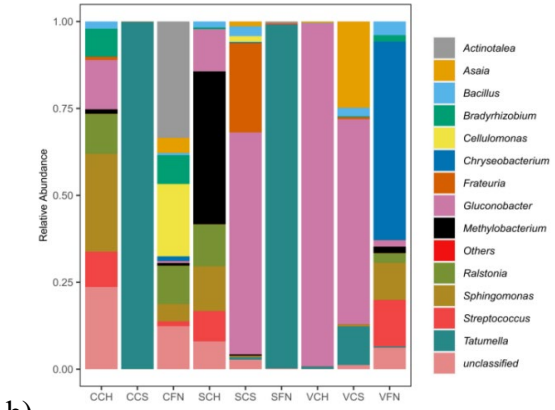
At the phylum level, the microbial composition represented by bacteria was classified into 16 phyla (Figure 3.5b), with 4 of them accounting for a relatively large proportion: *Proteobacteria* (75.3%), *Bacteroidetes* (7.9%), *Actinobacteria* (7.8%), and *Firmicutes* (7.6%). *Proteobacteria* have also been identified as dominant bacteria in other fermented foods. *Firmicutes* have been demonstrated to be an important core bacterial group in fermented foods, with their proliferation observed to be maximal during fermentation.

The microbial structure at the genus level is presented in the bar chart (Figure 3.6). In the case of fungi (Figure 3.6a), the most frequent genera found in all samples are *Hanseniaspora* (82.8%), *Saccharomyces* (7.1%), *Metschnikowia* (5.6%), *Mortierella* (2.0%), *Meyerozyma* (0.6%), *Torulaspora* (0.3%), *Saitozyma* (0.1%),

Erysiphe (0.1%), *Alternaria* (0.1%), *Aspergillus* (0.1%), *Russula* (0.1%), *Trichoderma* (0.05%), and *Mycosphaerella* (0.05%).



a)



b)

Figure 3.6. The 15 Most Frequent Genera: a) Fungi and b) Bacteria at the Genus Level.

For bacteria (Figure 3.6b), the most common genera are *Gluconobacter* (25.3%), *Tatumella* (23.4%), *Chryseobacterium* (4.6%), *Sphingomonas* (4.3%), *Asaia* (4.2%), *Methylobacterium* (4.2%), *Ralstonia* (3.2%), *Actinotalea* (3.0%), *Frateuria* (2.7%), *Streptococcus* (2.7%), *Cellulomonas* (2.5%), *Bradyrhizobium* (2.5%), and *Bacillus* (1.8%).

It is observed that *Mortierella* and *Saitozyma* have a negative correlation with *Erysiphe*, *Alternaria*, and *Mycosphaerella*—three plant pathogens—while *Hanseniaspora* also inhibits *Aspergillus*. *Meyerozyma*, along with most bacteria and fungi, are negatively correlated (Figure 3.7).

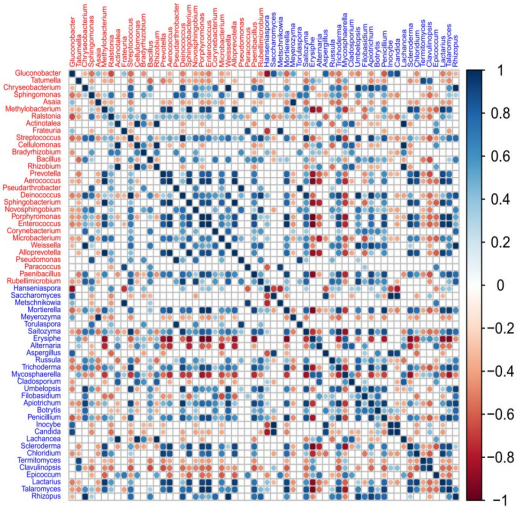


Figure 3.7. Pearson Correlation of the Top 30 Fungi and Bacteria.

The lack of protection against oxygen and slow fermentations will favor the development of acetic bacteria [18]. It can be observed in Figure 3.7 that *Saccharomyces* shows a positive correlation with *Gluconobacter*. The most attractive non-*Saccharomyces* yeasts, *Metschnikowia* and *Torulaspora*, do not correlate with other fungi, but they do exhibit positive correlations with some bacteria, among which the correlation coefficients between *Metschnikowia* and *Frateuria*, *Torulaspora* and *Pseudarthrobacter* are very high.

The occurrence of the species *Tatumella* shows a negative correlation with almost all other bacterial genera, indicating a possible inhibition of other bacteria by this genus. The LAB genus *Weisella* has a strong co-occurrence with *Chryseobacterium*, *Aerococcus*, and *Pseudarthrobacter*.

IV. IMPLEMENTATION OF BIOPROTECTION TECHNOLOGY AND ECOLOGICAL CERTIFICATION

4.1. Implementation of Bio-protection Technology in the Production of Red Wines under Ecological Certification

The comparison is made between the control group (group C1) and the bio-protection group (wine BP). *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* were used as bio-protection agents added at the beginning of the vinification process in the BP group.

Based on the physicochemical results presented in Table 4.1, the wines produced with non-*Saccharomyces* yeasts as bio-protection agents were characterized by a significantly higher mass concentration of volatile acids and total phenolic substances, total anthocyanins, and a predominant IPT.

The polyphenol and anthocyanin content in the control group is 899.95 mg/dm³ and 191.65 mg/dm³, relatively low values, which are associated with the grape variety used. In contrast, the BP group exhibits polyphenol and anthocyanin contents of 1312.35 mg/dm³ and 492.00 mg/dm³, respectively, which are significantly higher than those in group C1. The bio-protection technology considerably increased the anthocyanin and polyphenol content in the wines.

Table 4.1. Physicochemical Analysis of Wines Produced with Bioprotection

Physicochemical Parameters	C1 Group	BP Group
Alcohol content, % vol.	14.44±0.01a	14.49±0.04a
pH	3.61±0.01a	3.63±0.01a
Total acidity, g/dm^3	4.80±0.00a	4.65±0.07a
Volatile acidity, g/dm^3	0.21±0.01b	0.45±0.01a
Titratable acidity, g/dm^3	1.59±0.15a	1.51±0.06a
Lactic acid, g/dm^3	1.52±0.23a	1.65±0.01a
Total polyphenol index (TPI)	34.30±0.15b	46.98±0.06a
Total phenolic compounds,mg/dm^3	899.95±132.02b	1312.35±15.34a
Total anthocyanins, mg/dm^3	191.65±14.64b	492.00±3.39a

Thus, the increase in anthocyanin and polyphenol content suggests that wines from the BP group can maintain a more stable color, structure, and complexity during their maturation in oak barrels. The high level of polyphenols may contribute to a more pronounced astringency, a feature that was not observed in the sensory evaluation.

According to the radar chart presenting the sensory profile of the wines (Figure 4.1), the bio-protected wine received the maximum score in sensory characteristics, except for balance. The differences are small, except for the intensity of the aroma and the fruit aroma, with the bio-protected wines scoring better than the control ones.

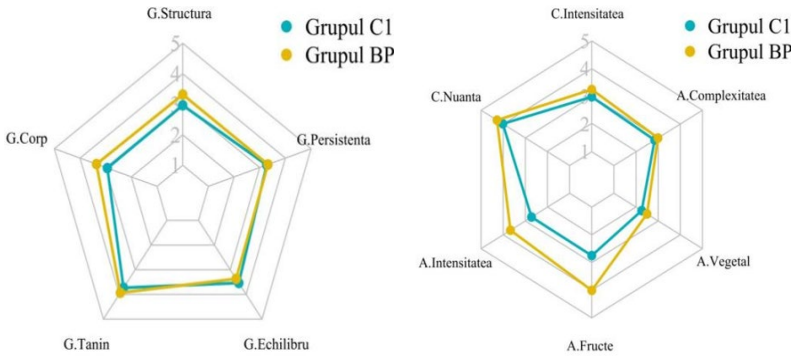


Figure 4.1. Radar Chart of Sensory Analysis of Bioprotected Wines

4.2 Implementation of the Process for Preparing Optimized Indigenous Yeast Must in the Production of Red Wines under Ecological Certification

The results of the physicochemical indices (Table 4.2) show that the MLI group exhibits significant differences in chemical composition, highlighting the unique metabolic characteristics of the non-*Saccharomyces* yeasts involved in the fermentation process. On an organoleptic level, all results for the MLI group did not show significant differences compared to the control group.

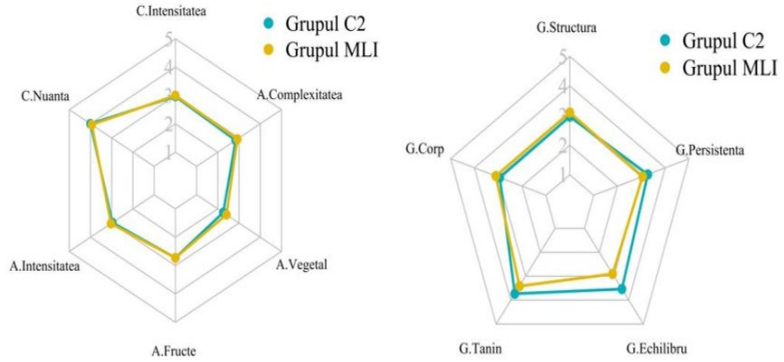


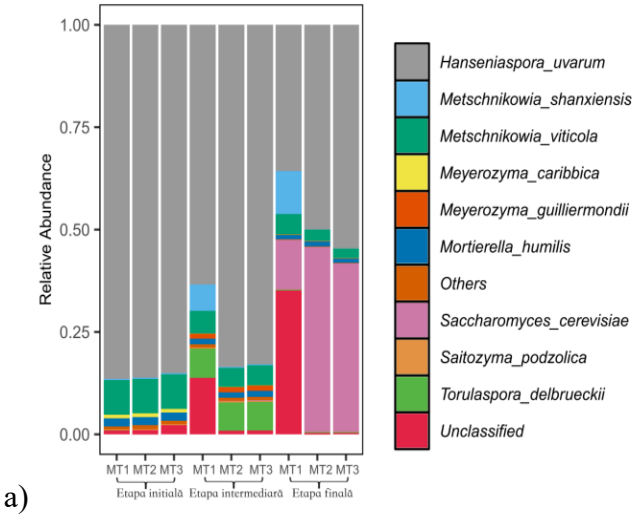
Figure 4.2. Radar Chart of Sensory Analysis of Wines with Traditional MLI

Acest lucru a fost confirmat de evaluarea senzorială, cu un număr mai mare de degustători care au observat defecte olfactive în MT1.The experimental study of the optimized MLI variant was conducted in the laboratory, and the vinification process was carried out according to the description in Section 2.2. During the spontaneous fermentation process for the preparation of MLI-T, it is evident that MT1 encountered problems around the middle of fermentation, indicated by an increased presence of unclassified fungi at the final stage of MT1, along with an insufficient amount of S. cerevisiae (Figure 4.3). This was confirmed by the sensory evaluation, with a higher number of tasters observing olfactory defects in MT1.

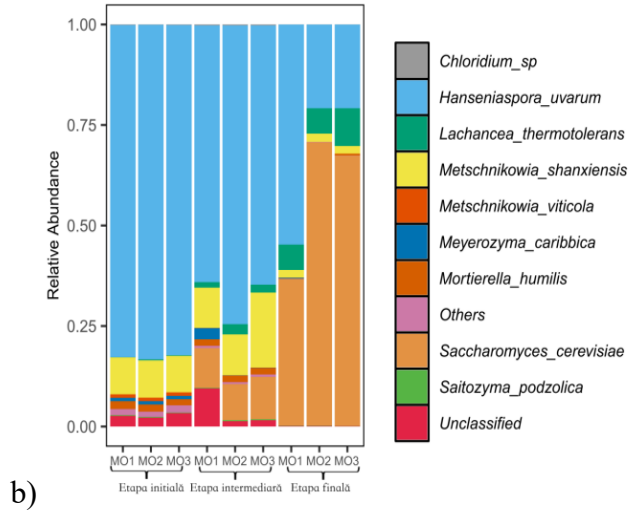
During alcoholic fermentation, the MO group had a slower start, but it registered a stable rate beginning on the second day, reaching almost completion by the fifth day. This suggests that the addition of wine inhibited the competition from undesirable microorganisms, thereby increasing the stability of fermentation. In contrast, the C3 group had an even slower start, possibly due to the need for reactivation and adaptation of the commercial S. cerevisiae. Although the fermentation rate increased in later stages, the overall efficiency remained lower than that of the MO and MT groups.

Tabelul 4.2. Physicochemical Analysis of wines with traditional MLI (C2-Control group, MLI-Indigenous Yeast Starter Variant)

Physicochemical Parameters	C2 Group	MLI Group
Alcohol content % vol.	14.44±0.01a	14.46±0.10a
pH	3.61±0.01a	3.68±0.01a
Volatile substances, g/dm^3		
acetaldehyde	4.35±1.20a	4.30±2.97a
ethyl acetate	60.55±0.35b	70.25±0.35a
methanol	67.95±2.05a	66.00±2.12a
isoropanol	2.00±0a	2.00±0.28a
n-propanol	68.60±0.57a	47.60±1.41b
iso-butanol	48.20±0.28a	45.90±0.57b
n-butanol	1.15±0.07a	0.60±0.14a
iso-pentanol	239.25±6.01b	244.45±5.87a
Glycerol content (g/dm^3):	5.40±0.14a	5.70±1.27a
2.3-butylene glycol content (g/dm^3):	164.15±11.95a	172.05±43.06a
Organic acid content (g/dm^3)		
tartric	1.59±0.15a	1.35±0.02a
malic	0.15±0.01a	0.13±0.01a
citric	0.13±0.01a	0.11±0a
succinic	0.45±0.11a	0.30±0.11a
lactic	1.52±0.23a	1.43±0.04a



a)



b)

Figure 4.3. Relative Abundance of the Top 10 Fungi in MLI-T Preparation a); and MLI-O Preparation b).

Similarly, during the preparation of MLI-O, MO1 encountered similar issues, with higher levels of unclassified organisms during the middle of fermentation compared to the other samples. Although the levels of *S. cerevisiae* were lower than in the

other two samples at the end of fermentation, they were still higher than in the MT group, a fact confirmed by the sensory evaluation. Although NGS could not identify the specific unclassified microorganisms, it is plausible that these organisms contributed to the alteration of the wine and the formation of unpleasant flavors.

Comparing the MLI preparation processes, it is evident that more non-*Saccharomyces* yeasts were involved in fermentation such as the species *Hanseniaspora uvarum* and *Metschnikowia* with *S. cerevisiae* representing less than 50%. This could be a contributing factor to the defects observed in the MT group samples.

In the preparation process of the MO group, with the exception of MO1, *S. cerevisiae* eventually dominated the microbial community. Initially, *Metschnikowia* participated in fermentation, gradually decreasing, while *Lachancea* increased. The MDI-O process, through the addition of alcohol, inhibited some non-*Saccharomyces* yeasts and other microorganisms that can alter the wine, ultimately increasing the quantity of non-*Saccharomyces* yeasts in the PdC, resulting in a safer and more efficient alcoholic fermentation process.

Regarding the physicochemical properties, significant differences were observed only in terms of volatile acidity and malic acid content. The levels of volatile acidity and malic acid in both experimental groups using PdC were significantly lower than those in the control group. In the PCA analysis described in Figure 4.4, two principal components explained 79.8% (55.6% + 24.2%) of the variation, indicating strong explanatory power. This PCA analysis is considered effective at this level of interdependence.

The sensory attributes highlight a significant advantage of the C group, with a tight distribution of samples within the group, indicating stability and consistency. The samples in the MO group (green triangle) are located mainly in the bottom right and central areas of the graph, where MO2 and MO3 are close to the "Fresh Fruit" and "Floral" attributes, highlighting strong fresh fruit characteristics and floral notes.

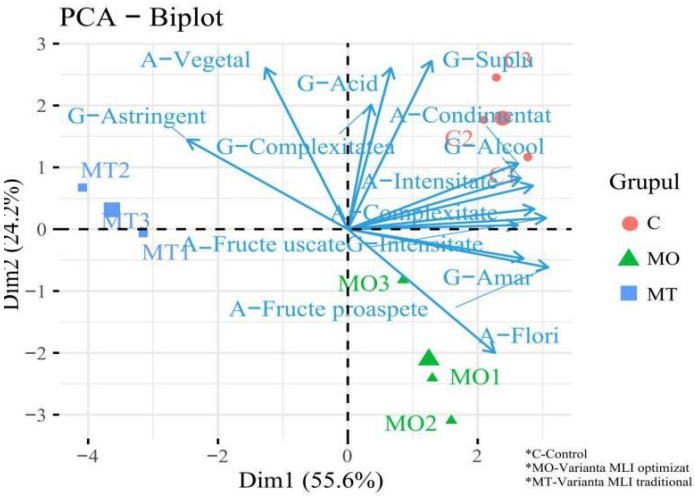


Figure 4.4. PCA Analysis of Sensory Evaluation

However, the MO group stands apart from the others and is associated with the "Bitter" attribute, which suggests possible sensory defects or high variability. The MT group samples (blue square) are located on the left side of the graph, associated with negative attributes such as "Vegetal" and "Astringent," reflecting inferior sensory quality and high taste variability among samples.

A total of 59 volatile compounds were identified, including 30 esters, 6 alcohols, 7 aldehydes, 3 ketones, 1 terpene, 6 acids, 2 thiols, 2 pyrazines, and 2 other compounds. Group C had the highest amount of positive volatile compounds, especially esters, contributing to a higher aroma intensity in the sensory evaluations. The MO group, although it had a slightly lower amount of positive volatile compounds than group C, still maintained a low proportion of negative volatile compounds. In contrast, the MT group had a lower number of positive compounds and notably negative compounds.

GENERAL CONCLUSIONS

1. The total number of yeasts on the grape surface ranges from 2.2E+04 to 3.9E+07 CFU/berry, while the number of culturable yeasts on the grape surface ranges from 4.1E+02 to 8.0E+06. In contrast, the must contains a lower number of yeasts than the grape surface, varying between 8.9E+02 and 4.25E+06 CFU/mL, and the number of culturable yeasts varies from 8.9E+02 to 9.0E+05 CFU/mL.

2. A statistical analysis of climatic factors and the use of phytosanitary products established that the products used to combat powdery mildew reduce the number of culturable yeasts, but contribute to an increase in the total number of yeasts. Conversely, the phytosanitary products for powdery mildew cause the yeasts on the grape surface to shift into a viable but non-culturable state, leading to a decrease in the number of yeasts in the must.

3. The IGP Valul lui Traian region was found to have the greatest microbial diversity among the three national IGP regions, as demonstrated by the NGS study.

4. At the phylum level, the fungal and bacterial composition of Moldovan grapes shows that the predominant fungi belong to *Ascomycota*, while the predominant bacteria belong to *Proteobacteria*.

5. At the genus level, the dominant fungi are *Hanseniaspora*, followed by *Saccharomyces* and *Metschnikowia*; for bacteria, the dominant genera are *Gluconobacter*, *Tatumella*, and *Chryseobacterium*, while lactic acid bacteria are the least prevalent.

6. The production of bio-protected wines was carried out using two non-*Saccharomyces* yeasts: *Metschnikowia pulcherrima* and *Torulaspora delbrueckii*, which are frequently found on Moldovan grapes. The resulting wine is of good quality, featuring a more pronounced fruit aroma bouquet and a higher content of phenolic compounds and anthocyanins.

7. The optimized MLI process produced wines with lower volatile acidity and malic acid compared to those obtained with

commercial yeasts, while also exhibiting a more pronounced fruity aroma bouquet, attributed to the presence of *Lachancea thermotolerans*.

PRACTICAL RECOMMENDATIONS

1. It is recommended to gradually reduce or replace chemical phytosanitary products with organic and biological protection solutions, such as microbe-based pesticides (for example, *Meyerozyma*, identified in subsection 3.2 as being present in indigenous microbial communities).

2. Select and use appropriate non-*Saccharomyces* yeasts for bioprotection: *M. pulcherrima* and *T. delbrueckii*, which have the ability to develop wines with a fruity aroma profile.

3. Select indigenous strains of *Saccharomyces* and non-*Saccharomyces* yeasts from the wine region to carry out alcoholic fermentation, enhance the wine's aromatic complexity, and support sustainable and durable winemaking practices.

4. Under stress conditions for indigenous microbial communities (such as during hot summer periods or cold nights before grape harvest), the use of optimized MLI would prove to be an effective and natural method, contributing to the efficiency and quality of alcoholic fermentation.

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- **Articole în reviste științifice peste hotare:**
  - ✓ în reviste din alte baze de date acceptate de către ANACEC, Baza DOAJ

1. Wang, F., **Yao, M.,** Arpentin, G. Sensory evaluation of Fetească Neagră wine in Republic Moldova. În: *Magarach Vinogradstvo i Vinodelie*, 2022, vol. 24(1), 90-94. ISSN 0236-1264. doi:10.35547/IM.2022.38.66.014

- **Articole în reviste științifice naționale acreditate:**
  - ✓ din Registrul Naţional al revistelor de profil Categoria B^+

2. Wang, F., **Yao, M.,** Arpentin, G. Exploring the Micro and Macro terroir of Feteasca Neagra wine from Moldova. În: *Journal of Engineering Sciences*, 2024, vol. 31, nr.1, pp. 97-111. ISSN 2587-3474. doi:https://doi.org/10.52326/jes.utm.2024.31(1).08

3. **Yao, M.** Microbial diversity on grape surface and its research status. În: *Journal of Engineering Sciences*, 2024, vol.30, nr. 2, pp.158-172. ISSN 2587-3474. https://doi.org/10.52326/jes.utm.2023.30(2).14

  - ✓ din Registrul Naţional al revistelor de profil Categoria B

4. **Yao, M.,** Wang, F., Arpentin, G. Studiul microorganismelor strugurilor din podgoriile Republicii Moldova: influența factorilor uman și natural. În: *Revista de Ştiinţă, Inovare, Cultură şi Artă „Akademos"*, 2023, vol.70, nr.3, pp. 99-106. ISSN 1857-0461. doi.org/10.52673/18570461.23.3-70.08

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8. **Yao, M.,** Wang, F., Arpentin, G. Bioprotection as a tool to produce natural wine: Impact on physicochemical and sensory analysis. În: *43rd World Congress of Vine and Wine*, Ensenada, Mexico, 2022, vol. 56, pp.1-4. ISSN 2273-1709. https://doi.org/10.1051/bioconf/20235602019

ADNOTARE

Meiling Yao, „Diversitatea microbiană a soiurilor de struguri din trei regiuni cu indicații geografice ale Republicii Moldova”, Teză de doctorat în științe inginerești, Chișinău, 2025

Structura tezei: Teza este compusă din introducere, patru capitole, concluzii și recomandări, bibliografie cu 186 referințe, 8 anexe, 122 pagini de conținut de bază, 21 tabele, 30 figuri. Rezultatele au fost prezentate în 8 publicații științifice.

Cuvinte-cheie: Diversitatea microbiană, factori climatici, managementul vitei de viei, tehnologia de secvențiere de nouă generație (NGS), indicații geografice protejate (IGP).

Scopul lucrării: constă în investigarea diversității microbiene a strugurilor din cele trei indicații geografice protejate a Republicii Moldova, explorarea funcției resurselor microbiene.

Obiectivele cercetării: Studierea cantitativă a microorganismelor strugurilor produși în 3 zone IGP din RM; studierea factorilor care influențează cantitatea și calitatea microorganisme-lor asociate strugurilor; identificarea microbiomului strugurilor din RM; dezvoltarea unei strategii inovatoare de vinificație prin utilizarea microorganismelor comerciale și/sau indigene.

Noutatea științifică și originalitatea tezei consta utilizarea, în premieră, a tehnologiei NGS în domeniul microbiologiei strugurilor produși în regiunile IGP din RM, precum și în folosirea, pentru prima dată la nivel mondial, a tehnologiei NGS pentru soiul de struguri autohton - Fetească Neagră.

Rezultatele obținute care contribuie la soluționarea unei probleme științifice importante: Descrierea compoziției comunității microbiene din mustul strugurilor moldovenești, demonstrând că speciile *Torulaspora delbrueckii* și *Metschnikowia sp.* sunt levuri non-*Saccharomyces* larg răspândite pe strugurii produși în zonele IGP din RM. Acest lucru contribuie la dezvoltarea produselor vitivinicole cu caracteristici locale prin utilizarea diferitelor resurse microbiene indigene.

Semnificația teoretică și valoarea practică aplicată: Pe baza cercetării efectuate, a fost propusa tehnologia producerii vinurilor cu bioprotecție folosind două levuri non-*Saccharomyces: Torulaspora delbrueckii* și *Metschnikowia pulcherrima*. Procedura de pregătire a maielei de levuri indigenă (MLI) a fost optimizată prin fortificarea cu vin a mediului de cultură, și a fost stabilit efectul acestui procedeu tehnologic asupra calității organoleptice și parametrilor fizico-chimice a vinurilor elaborate.

Implementarea rezultatelor științifice: Elementele tehnologice ce țin de bioprotecție și MLI au fost aplicate în practică la ÎM „Vinăria Purcari” SRL la producerea ecologică a vinurilor roze și roșii pe parcursul campaniilor de vinificație 2021-2023.

АННОТАЦИЯ

Meiling Yao «Микробное разнообразие сортов винограда из трех регионов с географическими указаниями Республики Молдова». Диссертация на степени доктора технических наук, Кишинев, 2025.

Структура диссертации: Диссертация состоит из введения, четырех глав, выводов и рекомендаций, библиографии из 186 источников, 8 приложений, 122 страницы основного содержания, 21 таблиц, 30 рисунков. Результаты исследования были представлены в 8 научных публикациях.

Ключевые слова: Микробное разнообразие, климатические факторы, управление виноградниками, технология нового поколения секвенирования (NGS), защищённое географическое указание (ЗГУ).

Цель работы: Исследование микробного разнообразия винограда из трех защищенных географических указаний (ЗГУ) РМ и изучение функций микробных ресурсов.

Задачи исследования: Количественное изучение микроорганизмов винограда, выращенного в трех регионах с ЗГУ в РМ; исследование факторов, влияющих на количество и качество микроорганизмов, связанных с виноградом; идентификация микробиота винограда из РМ; разработка инновационной стратегии виноделия с использованием коммерческих и/или местных микроорганизмов.

Научная новизна и оригинальность диссертации: Впервые была применена технология NGS для исследования микробиологии винограда, выращенного в регионах ЗГУ в РМ, а также впервые в мире технология NGS была использована для изучения местного сорта винограда – Fetească Neagră.

Полученные результаты, способствующие решению важной научной проблемы: Выявлен состав микробного сообщества в сусле молдавского винограда, продемонстрировано, что *Torulaspora delb.* и *Metschnikowia sp.* являются широко распространенными несахаромицетными дрожжами на винограде, выращенном в регионах ЗГУ в РМ. Это открытие способствует развитию винодельческой продукции с локальными характе-ристиками за счет использования различных местных микробных ресурсов.

Теоретическая значимость и прикладная практическая ценность: Была предложена технология производства вин с биозащитой с использованием двух несахаромицетных дрожжей: *Torulaspora delb.* и *Metschnikowia pul.* на основе проведенных исследований. Процедура подготовки местной дрожжевой закваски (МДЗ) была оптимизирована путем обогащения ферментационной среды вином, установлено влияние этого технологического процесса на органолептические и физико-химические свойства вин.

Внедрение научных результатов: Технологические элементы, связанные с биозащитой и закваской МДЗ, были применены в практике на предприятии КОО „Vinăria Purcari” для производства экологических розовых и красных вин в винодельческих кампаний 2021-2023 годов.

ABSTRACT

Meiling Yao "Microbial diversity of grape varieties from three Protected Geographical Indication regions of the Republic of Moldova." PhD thesis in engineering sciences, Chișinău, 2025.

Thesis structure: The thesis consists of an introduction, four chapters, conclusions and recommendations, a bibliography with 186 references, 8 annexes, 122 pages of core content, 21 tables, and 30 figures. The results have been presented in 8 scientific publications.

Keywords: Microbial diversity, climatic factors, vineyard management, next-generation sequencing technology (NGS), protected geographical indications.

Purpose of the work: To investigate the microbial diversity of grapes from the three protected geographical indications of the Republic of Moldova and to explore the function of microbial resources.

Research objectives: Study the quantity of microorganisms in grapes produced in the three protected geographical indications; study the factors that influence the quantity of grape micro-organisms; identify the microbial community of grapes in Moldova; develop an innovative winemaking strategy using commercial and/or indigenous microorganism.

Scientific novelty and originality of the thesis: the first application of NGS technology in the field of grape microbiology produced in the protected geographical regions of the Republic of Moldova, as well as the first worldwide use of NGS technology for the indigenous grape variety - Fetească Neagră.

Results obtained that contribute to solving an important scientific problem: Revealing the composition of the microbial community on the surface of Moldovan grapes, demonstrating that *Torulaspora delbrueckii* and *Metschnikowia sp.* are widespread non-*Saccharomyces* yeasts on grapes of the Republic of Moldova. This contributes to the development of viticultural products with local characteristics through the use of various indigenous microbial resources.

Theoretical significance and practical applied value: Based on the conducted research, a bioprotection wine technology using two non-*Saccharomyces* yeasts: *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, was proposed. The procedure for preparing an indigenous yeast starter (IYS) was optimized by fortification with wine in the culture medium, and its effect on the organoleptic and physicochemical quality of the final products was established.

Implementation of scientific results: Technological elements (bio protection and IYS) were applied in the practice of LLC „Vinăria Purcari" for the production of rosé and red wines in conversion to organic viticulture during the 2021-2023 campaigns.

YAO Meiling

DIVERSITATEA MICROBIANĂ A SOIURILOR DE STRUGURI DIN TREI REGIUNI CU INDICAŢII GEOGRAFICE ALE REPUBLICII MOLDOVA

253.03 - TEHNOLOGIA BĂUTURILOR ALCOOLICE ȘI NEALCOOLICE

Rezumatul tezei de doctor în ştiinţe inginerești

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