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THE IMPACT OF NON-SACCHAROMYCES YEASTS ON GRAPE MUST FERMENTATION: COMPREHENSIVE STUDY

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Abstract: *This study investigates the impact of non-Saccharomyces Torulaspora delbrueckii yeast on the fermentation process of grape must in the production of white dry wine, specifically focusing on two grape varieties, 'Aligote' and 'Chardonnay'. The fermentation was carried out using two different methods, co-inoculation and successive fermentation, both under laboratory and microvinification conditions. The physicochemical parameters of the resulting wines, such as total sugars, ethanol, volatile acidity, and total acidity, were analyzed. Sensory evaluation of the wines demonstrated that successive fermentation with the inoculation of Saccharomyces yeasts after reaching 3% vol. alcohol concentration contributed to an improved quality, complex aroma, and balanced taste of the wine, as indicated by high organoleptic scores. These findings suggest that the combination of Torulaspora delbrueckii yeast with indigenous yeast strains can enhance the overall quality of white dry wine.*

Key words: *Torulaspora delbrueckii, co-inoculation, successive fermentation, physicochemical parameters, sensory evaluation.*

1. Introduction

Wine, an alcoholic beverage, is produced through the fermentation of grape juice, where sugars undergo a series of biochemical reactions to transform into ethyl alcohol. Yeasts play a crucial role in initiating and directing the specific character and biochemical processes of fermentation. Additionally, the by-products of fermentation significantly contribute to shaping the overall flavour profile of wine, making them as essential as the primary product, ethyl alcohol [3].

The process of converting grape must into wine involves a diverse array of microorganisms, including yeasts, molds, and bacteria. Alcoholic fermentation, a vital stage of winemaking, is primarily carried out by yeasts. The microflora involved in natural fermentation originates from grape berries as well as

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the winery environment and equipment. The composition of yeast species on grape berries is influenced by various biotic and abiotic factors such as grape variety, weather conditions, and viticultural practices [6, 15, 16, 29]. Non-*Saccharomyces* yeasts, including *Hanseniaspora, Candida, Kluyveromyces, Metschnikowia, Pichia, Cryptococcus,* and *Rhodotorula*, are predominantly found on grapes, while Saccharomyces genera are less prevalent [16]. Although non-*Saccharomyces* yeasts initiate fermentation and develop during the early stages, their population diminishes rapidly, allowing *Saccharomyces cerevisiae* (*S. cerevisiae*) to dominate until the end of alcoholic fermentation.

Several studies have demonstrated the positive impact of certain non-*Saccharomyces* yeasts on the aroma, sensory complexity, and colour stability of the final wine product [1, 2, 5, 7, 19-21, 27, 28]. *Torulaspora delbrueckii* has garnered attention due to its excellent fermentation performance, characterized by low levels of ethanol, higher alcohols, volatile fatty acids, and volatile acidity. Furthermore, it contributes significantly to the aromatic profile of wine [10-12, 17].

Studies [4, 12, 18] indicate a positive synergistic effect between *T. delbrueckii* and *S. cerevisiae* when co-inoculated in higher proportions during fermentation, resulting in improved aromatic properties of the wine. Specific volatile compounds contributed by T. delbrueckii have been associated with the fruity character of the wine.

The use of non-*Saccharomyces* yeasts in winemaking has gained momentum, with initial applications focusing on enhancing the aromatic profile using widely available strains such as Biodiva™ Td291™ (Lallemand, Blagnac Cedex, France) and Prelude™ (Chr. Hansen, Hoersholm, Denmark). Other non-*Saccharomyces* yeast strains, including *S. pombe* reticulated in alginate beads for demalication (Proenol SA, Canelas, Portugal), *L. thermotolerans* (formerly *Kluyveromyces thermotolerans*), and *P. kluyveri* (Chr. Hansen, Hoersholm, Denmark), have also gained recognition [13]. These strains are often combined with *S. cerevisiae* to ensure complete sugar depletion and achieve the desired level of wine dryness. Primary commercial non-*Saccharomyces* yeast species have been extensively described by Morata and Suárez-Lepe [14].

The aim of our research is to assess the impact of *Torulaspora delbrueckii* yeast on the fermentation of grape must in white dry wine production using indigenous yeast strains. It examines the effects of co-inoculation and successive fermentation methods on the wine's physicochemical parameters and sensory qualities. The goal is to determine if these methods improve the wine's overall quality, aroma complexity, and taste balance.

2. Materials and Methods

2.1. Laboratory Conditions

The research was conducted at the Laboratory of Biotechnology and Wine Microbiology, located at the Scientific-Practical Institute of Horticulture and Food Technologies in the Republic of Moldova. The study utilized grapes from the 'Aligote' cultivar, which were harvested at an appropriate technological maturity with a sugar content of 218 g/L. The grapes were processed following the classical technology for white wine production in the laboratory conditions.

The fermentation of the grape must was carried out in 3-liter tanks, and specific conditions were provided to ensure proper alcoholic fermentation. The must was cleared, clarified, and sulfated to eliminate spontaneous microbiota. Selected yeasts, including non-*Saccharomyces Torulaspora delbrueckii* (Enartis FERM, Italy) and local yeast strains (CNMN-Y-26) isolated from the 'Chishinau' wine center, were introduced into the tanks in quantities that ensured the optimal density of yeast cells/mL. Two fermentation schemes were employed: co-inoculation, where non-*Saccharomyces* (10^5 CFU/mL) and *Saccharomyces* yeasts (10^6 CFU/mL) were inoculated simultaneously, and successive fermentation, with inoculation of non-*Saccharomyces* strain (10^5 CFU/mL) and a *Saccharomyces* yeasts (10^6 CFU/mL) after reaching an alcohol concentration of 3% vol.

2.2. Microvinification Conditions

In the second phase of our research, experiments were conducted under microvinification conditions using 10-liter tanks. The must for fermentation, derived from 'Chardonnay' grapes, was cleared, clarified, and sulfated to eliminate spontaneous microbiota. The initial sugar concentration was 220 g/L, and the mass concentration of titratable acidity was 8.2 g/L. The control group of monocultures consisted of Active Dry Industrial Yeast (Oenologia LB8, Germany), indigenous CNMN-Y-26 yeast strains, and non-*Saccharomyces* yeasts (Torulaspora delbrueckii, Enartis FERM, Italy), which were introduced into the tanks in quantities that ensured the optimal density of yeast cells for the fermentation process (10^6 CFU/mL). Therefore, to determine the optimal inoculation time for *Saccharomyces* yeasts, a scheme of successive alcoholic fermentation was chosen, and the inoculation of *Saccharomyces* yeasts (10^6 CFU/mL) took place when the alcohol concentration in the medium reached 3% vol. and 6% vol.

A chemical analysis was performed to assess various parameters. The total sugars content (g/L) in the musts was determined using the areometric method according to SM GOST 13192-73 [23]. The mass concentration of non-fermented sugars (g/L) in wines was determined by the indirect titration method specified in SM GOST 13192-73 [8]]. The concentration of ethanol (% vol.) was determined by distillation following SM GOST 51653:2010 [25]. Volatile acidity was measured by titration of the volatile acids separated from the wine through steam distillation and titration of the distillate, as per SM GOST 51654:2012 [26]. The total acidity was determined by titration with bromthymol blue as an indicator, in accordance with SM GOST 51621:2008 [24]. Gas chromatography was employed to analyze wine aroma compounds by direct injection, as per SM 152:2020 [22]. All determinations were performed in triplicate.

A sensory analysis was conducted with a panel of 17 expert enologists in accordance with institutional regulations for tasting alcoholic beverages, using a rating scale from 0 to 10, to evaluate wines produced using *Saccharomyces* and non-*Saccharomyces* yeasts at the Scientific-Practical Institute of Horticulture and Food Technologies. The evaluation included assessments of color, taste, aroma, and typicality of the wines.

Statistical analyses were carried out using a one-way analysis of variance (ANOVA) to assess differences in physicochemical parameters between the musts and the wines. The analysis was performed using GraphPad Prism 5.0 software and an online calculator available at http://math.semestr.ru/ [9].

3. Results and Discussion

During the study, the fermentative activity of different yeast cultures and their combinations was determined under laboratory conditions, considering both mixed-combined and mixed-successive fermentation approaches. The results (Figure 1) demonstrated that all cultures and their combinations exhibited enhanced fermentation activity, except for the pure non-*Saccharomyces* strain, which displayed a reduced ability to ferment. This observation can be attributed to the characteristics of the *Torulaspora delbrueckii* yeast strain, which is known to have lower tolerance to high alcohol concentrations, resulting in a reduced capacity to ferment carbohydrates. According to the findings depicted in Figure 1, it is evident that active fermentation occurs when utilizing the indigenous yeast strain CNMN-Y-26. This particular strain effectively consumes all sugars present in the must within 20 days of inoculation, in stark contrast to the non-*Saccharomyces* yeast strain under investigation.

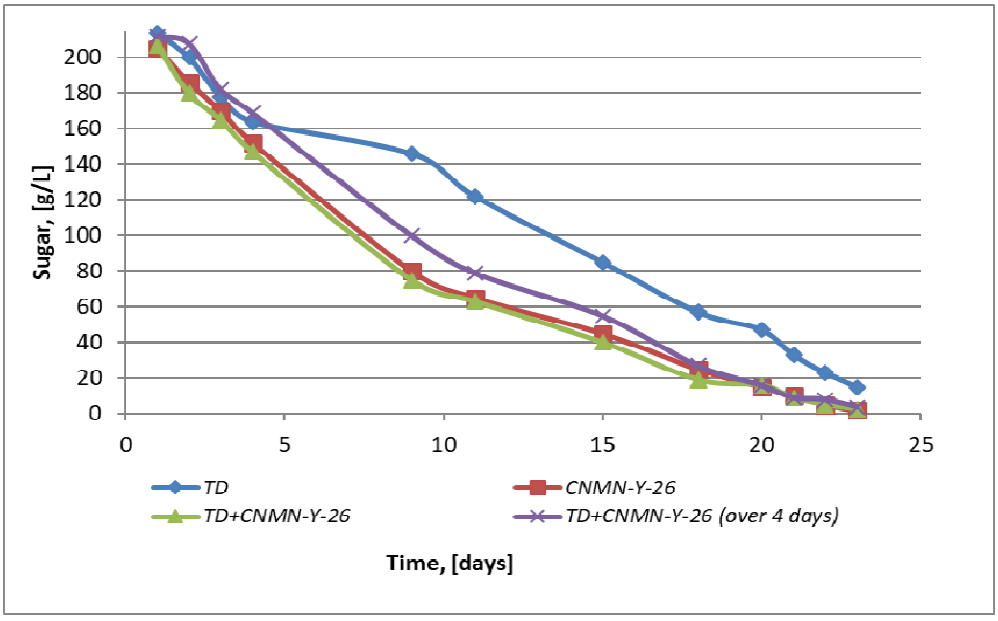

Fig. 1. *Sugar fermentation dynamics in 'Aligote' grape must using Torulaspora delbrueckii and CNMN-Y-26 yeast strains*

In the case of mixed-combined fermentations, where both non-*Saccharomyces* and indigenous yeast strains are co-inoculated, as well as in the case of mixed-successive fermentation, where non-*Saccharomyces* and indigenous yeast strains are sequentially inoculated once the alcohol concentration reaches 3% vol., a consistent and complete reduction in sugar content is observed. This suggests

that both fermentation approaches lead to successful sugar consumption and conversion during the process.

It is worth noting that the reduced fermentative ability of the pure non-*Saccharomyces* strain does not necessarily imply a negative impact on the overall fermentation process.

Non-*Saccharomyces* yeasts, such as *Torulaspora delbrueckii*, can contribute positively to the sensory profile and aromatic complexity of the resulting wine, despite their limited ability to fully convert sugars into alcohol. Their presence during the initial stages of fermentation can lead to the production of desirable flavour compounds and contribute to the overall sensory experience of the wine [1, 11].

To overcome the limitation of non-*Saccharomyces* yeasts in terms of complete fermentation, a co-inoculation approach involving the simultaneous inoculation of non-*Saccharomyces* and *Saccharomyces* yeasts has been employed in winemaking. This approach aims to take advantage of the positive attributes of both yeast types. The co-inoculation of *Torulaspora delbrueckii* with *Saccharomyces cerevisiae* has been shown to promote a positive synergistic effect, resulting in improved aromatic properties and overall quality of the wine [18].

The findings of this study were supported by other researchers who have highlighted the importance of non-*Saccharomyces* yeasts, particularly *Torulaspora delbrueckii*, in shaping the fermentation process and the sensory characteristics of the final wine product. The inclusion of non-*Saccharomyces* yeasts in winemaking has gained attention due to their potential to enhance the aroma, sensory complexity, and colour stability of wines. By carefully selecting and combining yeast strains, winemakers can optimize the fermentation process to achieve desired outcomes in terms of aroma, flavour, and overall quality [12, 17, 18].

Further investigations are necessary to explore the specific metabolic activities and interactions of different yeast strains during fermentation. Understanding the intricate dynamics between non-*Saccharomyces* and *Saccharomyces* yeasts can provide valuable insights into improving winemaking practices and tailoring wine characteristics to meet consumer preferences.

After the completion of alcoholic fermentation, the resulting dry white wines obtained through different fermentation schemes were subjected to thorough physicochemical analysis. The obtained results are presented in Table 1, providing valuable insights into the composition and characteristics of the wines.

According to the findings presented in Table 1, the white wine fermented using *Torulaspora delbrueckii* yeast strains in combination with selected yeasts demonstrates a higher alcohol concentration of 12.5% vol. (*Torulaspora delbrueckii*+CNMN-Y-26). On the other hand, the sequential inoculation of indigenous yeasts contributes to a lower alcohol content of 12.1% vol. This confirms that *Torulaspora delbrueckii* yeast strain has the ability to convert some sugars into secondary compounds, thereby enhancing the quality of the wine.

The titratable acidity concentration in the white wines obtained under laboratory conditions shows insignificant variation depending on the fermentation scheme used, ranging from 7.0 g/L to 7.5 g/L.

The pH values of the white wine samples obtained fall within a narrow range of 3.13 to 3.18.

Table1

Physico-chemical and organoleptic characteristics of dry white 'Aligote' wines obtained using different fermentation schemes

No.	Strain yeast	Alcohol [% vol.]	Total acidity [g/L]	Volatile acidity [g/L CH_3COOH]	Non-fermented sugars [g/L]	pH	Organoleptic assessment [0-10]
1	*Torulaspora delbrueckii*	11.80 ± 0.30	7.50 ± 0.20	0.50 ± 0.03	15.00 ± 1.10	3.15	7.60
2	CNMN-Y-26	12.90 ± 0.45	7.00 ± 0.10	0.40 ± 0.02	1.60 ± 0.10	3.13	7.95
3	*Torulaspora delbrueckii*+ CNMN-Y-26	12.50 ± 0.35	7.20 ± 0.10	0.40 ± 0.02	2.50 ± 0.20	3.18	7.90
4	*Torulaspora delbrueckii*+ CNMN-Y-26 (over 4 days)	12.10 ± 0.20	7.20 ± 0.20	0.33 ± 0.03	3.90 ± 0.35	3.15	8.10

The mass concentration of volatile acidity varies more significantly in the white wines obtained, ranging from 0.33 to 0.50 g/L. This variation can be attributed to the occurrence of different enzymatic reactions during fermentation.

The residual sugar values in the white wines do not exceed the allowable limits for this wine category, except for the wine produced using the non-Saccharomyces yeast strain *Torulaspora delbrueckii*, which reaches 15.0 g/L.

The sensory analysis of the wines reveals that the successive fermentation with the inoculation of *Saccharomyces* yeasts on the fourth day (alcohol concentration in the medium at 3% vol.) contributes to an improvement in quality, resulting in a wine with a complex aroma and balanced taste. This is further supported by the high organoleptic score of 8.1 points (*Torulaspora delbrueckii*+CNMN-Y-26 over 4 days).

During the winemaking campaign and under microvinification conditions, a comparative study was performed to evaluate the use of non-Saccharomyces and Saccharomyces yeasts in alcoholic fermentation. Previous research conducted at the laboratory scale demonstrated the positive effect of successive alcoholic fermentation on the quality of dry white wines.

Upon completion of the alcoholic fermentation, the wines obtained using different fermentation schemes underwent physicochemical and organoleptic analysis. The results of these analyses are presented in Table 2.

Based on the results presented in Table

2, it can be observed that white wines fermented using the *Torulaspora delbrueckii* yeast strains exhibit a lower alcohol concentration of 12.6% vol. On the other hand, the successive inoculation of the yeast leads to wines with a higher alcohol content of 12.9% vol. This confirms that *Torulaspora delbrueckii* is a yeast culture that is not resistant to high alcohol concentrations and demonstrates a reduced ability to ferment carbohydrates.

The titratable acidity concentration in the wines obtained under microvinification conditions shows insignificant variation depending on the fermentation scheme used, ranging from 7.7 g/L to 8.0 g/L. Similarly, the pH values of the white wine samples fall within a narrow range of 3.13 to 3.15.

Table 2

Physico-chemical and organoleptic characteristics of dry white 'Chardonnay' wines obtained using Saccharomyces and non-Saccharomyces yeasts under microvinification conditions

No.	Strain yeast	Alcohol [% vol.]	Total acidity [g/L]	Volatile acidity [g/L CH_3COOH]	Non-fermented sugars [g/L]	pH	Organoleptic assessment [0 – 10]
1	ADIY (control)	13.00 ± 0.50	7.70 ± 0.10	0.36 ± 0.04	1.60 ± 0.20	3.12	7.90
2	CNMN-Y-26 (control)	12.90 ± 0.40	7.90 ± 0.10	0.36 ± 0.04	2.40 ± 0.10	3.15	7.90
3	*Torulaspora delbrueckii* (control)	12.60 ± 0.30	8.00 ± 0.20	0.30 ± 0.03	7.60 ± 0.40	3.13	7.85
4	TD+ CNMN-Y-26 (3%vol.alcohol)	12.90 ± 0.40	8.00 ± 0.20	0.30 ± 0.03	3.30 ± 0.10	3.12	7.95
5	TD+ CNMN-Y-26 (6%vol.alcohol)	12.90 ± 0.40	8.00 ± 0.10	0.30 ± 0.02	3.40 ± 0.20	3.15	7.85
6	TD+ADIY (3%vol.alcohol)	12.90 ± 0.35	7.90 ± 0.10	0.30 ± 0.03	3.60 ± 0.10	3.14	7.95
7	TD+ADIY (6%vol.alcohol)	12.80 ± 0.45	8.00 ± 0.20	0.30 ± 0.02	3.90 ± 0.20	3.13	7.85

Note: TD- *Torulaspora delbrueckii*; ADIY-Active dry industrial yeast

The mass concentration of volatile acidity varies among all white wines obtained, ranging from 0.13 to 0.20 g/L. This variation can be attributed to the different enzymatic reactions that occur during fermentation. It is noteworthy that the use of *Saccharomyces* and *Torulaspora delbrueckii* yeasts for successive

fermentation contributes to the reduction of acetic acid, which is reflected in the mass concentration of volatile acids.

The residual sugar values in the white wines do not exceed the permissible limits for this wine category, except for the wine produced using the non-*Saccharomyces* yeast strain *Torulaspora delbrueckii*, which has a value of 7.6 g/L.

A comparative analysis of the aromatic content of 'Chardonnay' dry white wine was conducted using different fermentation schemes, which led to the identification of significant differences. The results presented in Table 3 highlight the impact of different fermentation schemes on the final content of volatile compounds in the white wines.

Table 3

Volatile substances content [mg/L] in dry white wines 'Chardonnay'

Compounds		Acetic aldehyde	Ethyl acetate	Propanol -1	Isobutanol	Sum of Amyl alcohols	Phenylethanol
Strain yeast	ADIY (control)	10.40 ± 0.69	35.90 ± 3.45	30.50 ± 4.11	23.90 ± 3.23	95.60 ± 5.36	49.50 ± 4.31
	CNMN-Y-26 (control)	6.80 ± 0.38	37.50 ± 3.75	29.40 ± 3.62	20.50 ± 3.13	100.10 ± 5.47	54.60 ± 4.54
	Torulaspora delbrueckii (control)	10.50 ± 0.73	35.50 ± 3.56	45.10 ± 4.25	21.60 ± 3.42	63.50 ± 4.23	70.20 ± 4.41
	TD+ CNMN-Y-26 [3%vol.alcohol]	7.70 ± 0.45	18.90 ± 1.25	66.40 ± 4.89	21.50 ± 3.44	75.80 ± 4.56	62.30 ± 3.21
	TD+ CNMN-Y-26 [6%vol.alcohol]	8.30 ± 0.51	19.10 ± 1.56	59.40 ± 4.23	20.70 ± 3.23	85.60 ± 5.42	59.20 ± 4.09
	TD+ ADIY [3%vol.alcohol]	8.50 ± 0.56	17.80 ± 1.16	71.60 ± 4.89	22.70 ± 3.77	77.90 ± 5.42	60.10 ± 3.89
	TD+ ADIY [6%vol.alcohol]	8.90 ± 0.61	18.10 ± 1.63	63.90 ± 4.29	23.50 ± 3.78	82.50 ± 5.78	55.80 ± 3.44

The findings in Table 3 indicate that the acetaldehyde production was low for all strains, with CNMN-Y-26 exhibiting the lowest level among the three control yeasts and their combination. Ethyl acetate concentrations showed no significant differences among the three control strains, but their combination resulted in a lower concentration, approximately half of the individual strains. The sum of amyl alcohols was lower for TD (control), while propanol-1 production was higher for the combination TD+CNMN-Y-26 (3% vol. alcohol) and TD+ADIY (3% vol. alcohol). The isobutanol production showed no significant differences among the strains and their combination. However, the concentration of 2-phenyl ethanol, with its distinctive rose-like aroma, was significantly higher for TD (control) and the TD+CNMN-Y-26 combination compared to ADIY (control) and CNMN-Y-26 (control).

The sensory analysis of the wines obtained revealed that successive fermentation with the inoculation of *Saccharomyces* yeasts upon reaching an alcohol concentration of 3% vol. significantly improves the overall quality of the wines, resulting in a complex aroma profile and a well-balanced taste. This is further supported by the high organoleptic scores of 7.95 points obtained in Table 2. On the other hand, successive alcoholic fermentation with the inoculation of *Saccharomyces* yeasts at an alcohol concentration of 6% vol. does not significantly influence the organoleptic properties as compared to the control samples, but it falls short when compared to the previous variant (3% vol.).

4. Conclusions

The physicochemical and sensory analysis of the dry white wines produced from 'Aligote' and 'Chardonnay' grape varieties using non-*Saccharomyces* yeasts highlights the positive impact of successive fermentation with the inoculation of *Saccharomyces* yeasts at an alcohol concentration of 3% vol. This fermentation approach contributes to enhancing the quality of the wines by promoting the development of complex aromas and achieving a harmonious and balanced taste.

These findings provide valuable insights into the characteristics and potential applications of non-*Saccharomyces* yeasts in wine production, further enriching our understanding of their behaviour throughout the winemaking process.

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