Investigation of using different specified yeasts and early protein stabilization for Tokaji dry wines

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Abstract. The use of special-purpose yeasts is becoming increasingly important in winemaking practice due to counterweight negative effects of climate change. There are no specified commercially available yeasts for most autochthonous grape varieties in the wine region of the world, just as in the case of Kövérszőlő, which plays an important role in the Tokaj wine region. The world's winemaking practices are increasingly shifting towards making new wines as quickly as possible in a given vintage, with a lower quantity, more conscious use of chemicals and minimising sulphurisation in the interests of sustainability. There are bentonite materials on the market with low-iron granulate that can be added to the must, removed with the lees at the end of fermentation and used to obtain a new wine with a clean smell and high purity of stable white must. In this study, different special yeast products of Erbslöh were tested in comparison with spontaneous fermentation in the important autochthonous variety Kövérszőlő. At the same time, the influence of simultaneous and early protein stabilization was analyzed. The aim was to explore the changes in analytics and sensor technology and the adaptation to the current market requirements.

1 Introduction

1.1 Tokaj Wine Region and "Kövérszőlő" grape varitey

The historical Tokaj Wine Region covering 5,500 ha vineyards in northeast Hungary has been a UNESCO World Heritage region since 2002. Produced from "noble rotted" grapes, Tokaji Aszú is known all over the world as one of the oldest botrytized wines. Special microclimatic conditions (due to the Bodrog and Tisza rivers, Indian summer), soil conditions (clay, loess on volcanic bedrock) and grape varieties of the Tokaj region offer favourable parameters to the formation of noble rot caused by *Botrytis cinerea*. The grapes undergo complex chemical modifications as the joint result of the enzymatic activity of *Botrytis* and the physical process of concentration [1].

Historically Tokaj had dozens of grape varieties (red ones too). After Phylloxera (early 20th century) white grapes became widespread: mainly Furmint (65%) and Hárslevelű (20%) while the rest is Sárgamuskotály, Kövérszőlő, Zéta (Furmint x Bouvier) and Kabar (Hárslevelű x Bouvier). Only these 6 varieties are allowed to be used for PDO (protected designation of origin Tokaji OEM wines). Kövérszőlő is an autochtonous grape variety with large berry size (Fig. 1) hence the name meaning fat. It is not currently widely grown in the Tokaj Wine Region, although it is a traditional variety. It is late-ripening, but it is still a week or two ahead of Hárslevelű and Furmint. Noble rot can progress very well, but the vine is sensitive to the location of the plantation; in prolonged humid conditions its fruits deteriorate very quickly. Rot-sensitive grape variety [2].

1.2 Specified yeast strains vs spontaneous fermentation in the case of autochtoneous grape variety

Indigenous yeasts, which naturally exist in grape tissues, have a major impact on the regulation of grape health, growth and yield [3]. Complex and genetically diverse yeasts are subsequently released into grape must/juices and their population dynamically changes during the wine fermentation process [3]. The many yeasts present during fermentation play a crucial role in wine production both through alcoholic fermentation and the release of desirable secondary metabolites that potentially enhance the complexity of the wine aroma [4,5]. Distinctive wines with a unique regional character can be controlled by indigenous yeasts, highlighting the importance of these microbes in adding economic and cultural value to wine [3]. The selection, maintenance and production of native yeast strains as dried cultures is a very costly and time-consuming process. Measures to counteract the negative

effects of climate change are more urgent and there is an increased interest in commercially available yeast preparations that are multi-component and contain wild yeasts. Their use in autochthonous varieties could be fruitful for several wine regions in the world [6].

In addition to there is a need to choose such a specified yeast owning many advantages during fermentation and having degradation products with potential beneficial effect on human health such as phenolic acids [7,8].

1.3 The essential of phenolic acid producing activity of yeasts

Phenols are compounds that have at least one phenyl ring. Phenols can be divided into flavonoids, coumarins, hydoxycinnamic acids, stilbenoids, monolignols, and condensed and hydrolyzable tannins. Cinnamic acid derivatives are a subgroup of phenolic compounds. Hydroxycinnamic acids (HCAs) are comprised mostly of phenolic acids but could also include monolignols, coumarins, and other downstream compounds except the flavonoids and stilbenoids [9]. The cinnamic acid derivatives without condensed skeleton (p-coumaric acid, ferulic acid, caffeic acid) are mostly present in the form of esters with tartaric acid (cutaric acid, ferulic acid, caffeic acid), of which caffeic acid (caffeyl tartrate) in particular is a preferred substrate for polyphenol oxidase enzymes in grapes [10]. An overview of phenolic pathways is shown in Fig. 2.

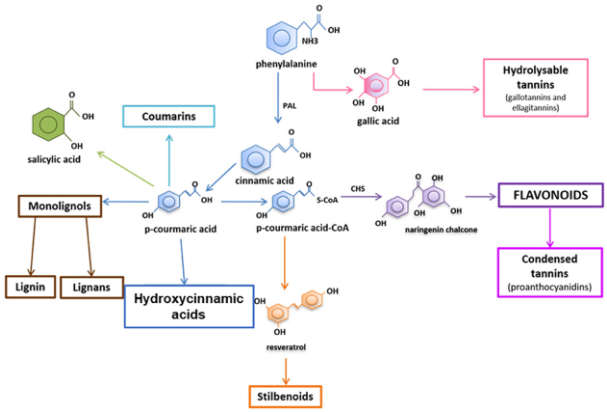

Figure 2. Phenolic pathway (Source: [9]).

Infection of grapes by Botrytis cinerea can cause an measurable increased value in the levels of caffeic acid, ethyl caffeate, ethyl gallate, fertaric acid, gentisic acid and quercetin [11].

They are readily absorbed and have beneficial physiological effects: antioxidant, reduction of plasma LDL cholesterol, improvement of lipid composition, reduction of arthritis, memory improvement [12].

1.4 Protein stabilization

The most common causes of turbidity in wines are oxidative changes, biological turbidity, crystal formation, metal precipitation and protein turbidity. Proteins are highly unstable chemical compounds and can very quickly make wines porous, cloudy and then turbid. The most common causes of problems caused by protein precipitation:

- oxygen in the air precipitates nitrogen-containing compounds;
- changes in wine temperature;
- shaking and transport of wine;
- the original balance between protein colloids and tannins is broken down during the cuvée making process
- changes in pH and/or alcohol content [10].

In order to prevent all this, efforts should be made to stabilise the proteins in musts in order to protect the fermentation aromas by removing the proteins that cause turbidity at the beginning of fermentation and to obtain a wine with a cleaner aroma and taste if the oxidase enzymes in the grapes are inactivated early in the winemaking process.

FermoBent® PORE-TEC (Erbslöh): it has a very fine powder structure and is manufactured using the PORE-TEC process, which allows for a special punched-sponge surface design, easier dissolution and suspension. It has a very low iron content and can be removed with the broom at the end of fermentation. Recommended but not required for higher adsorption efficiency, it can provide uniform CO_2 removal during fermentation, settles quickly at the end of fermentation. Depending on variety and vintage, the recommended dosage is 100-200 g/hl.

2 Materials and methods

2.1 Kövérszőlő must sample and treatments used

Harvest date: 06 September 2022.The raw material was infected by Botrytis at 30%. The initial sugar content was 224 g/l.

Figure 1. Kövérszőlő grape variety (Photo: Bene, 2023).

Three different Erbslöh specified yeast product preparations were used for the fermentation of a Tokaj autochthonous grape variety (Kövérszőlő) must: **Oenoferm® Wild&Pure** (Torulaspora delbrueckii+Saccharomyces strains), **Oenoferm® X-treme** (Saccharomyces cerevisiae+Saccharomyces bayanus), **Oenoferm® Freddo** (Saccharomyces cerevisiae var.bayanus). All batches of yeast strains were

simultaneously treated with bentonite called **FermoBent® PORE-TEC**. Vitamon® Liquid nutrient was added in any cases and VitaDrive®F3 biological nutrients for yeast rehydration was applied in samples with specified yeast products. Treatments used are shown in Table 1. Each experimental items were implemented in glass vessel of 10 litre and with 15 ºC without temperature control.

Table 1. Treatment used (WP-O.Wild&Pure; X-O.X-treme; F-O.Freddo).

Batches	Spontaneous	WP1	WP2	X1	X2	F1	F2
Yeast addition	-	30 g/hl	30 g/hl	40 g/hl	40 g/hl	40 g/hl	40 g/hl
Nutrient	200 ml/hl Vitamon Liquid	200 ml/hl Vitamon Liquid+20 g/hl VitaDriveF3	200 ml/hl Vitamon Liquid+20 g/hl VitaDriveF3	200 ml/hl Vitamon Liquid+20 g/hl VitaDriveF3	200 ml/hl Vitamon Liquid+20 g/hl VitaDriveF3	200 ml/hl Vitamon Liquid+20 g/hl VitaDriveF3	200 ml/hl Vitamon Liquid+20 g/hl VitaDriveF3
Fermobent	-	-	150 g/hl	-	150 g/hl	-	150 g/hl
SO_2	10 mg/l	10 mg/l	10 mg/l	10 mg/l	10 mg/l	10 mg/l	10 mg/l

2.2 Wine profiling

Examination of chemical composition was carried out with analysis (NMR - Nuclear Magnetic Resonance) in Diagnosticum Zrt. laboratory, Szerencs. H NMR Technique [13]: ^{1}H NMR (proton nuclear magnetic resonance) spectra acquisitions were performed on a 400 MHz spectrometer (Bruker Avance) and a magnet Bruker AscendTM 400 MHz equipment with 2H "lock" channel and z gradient at a frequency of 400.13 MHz and 26.85 °C.

The data analysis is performed at Bruker BioSpin GmbH (Rheinstetten, Germany) according to testing method AA-72-02-10 (Wine-Profiling 4.0.8), released on 08-Jul-2022 (DIN EN ISO/IEC 17025:2018 Accreditation Certificate D-PL-19229-01-00 of Bruker BioSpin GmbH).

NMR-based metabolomics has been used extensively to study wine fermentation and evaluate the fermentative characteristics of different yeast strains [14,15].

The ethanol and sugar contents were followed by Lyza-5000 Wine FTIR-analyzer, serial number: 82901790 and 2.0.0 software version.

STATA v17.0 software was used for statistical analysis. It is important to emphasize that the results obtained in the experimental set-up are not parallel fermentations per treatment, but each treatment is a batch and these have been compared by repeating the measurements in triplicate (n=3). All data are expressed as the mean ± standard deviation (SD).

2.3 Measurement of metals (copper and iron)

Copper: turbidimetric method, the increase of the absorbance is directly proportional to the concentration of copper in the sample according to REF984628 published by Thermo Fisher Gallery method and procedures [16]. Iron: colorimetric method, according to REF984326 published by Thermo Fisher Gallery method and procedures [16].

2.4 Examination of protein stability

According to OIV-MA-AS2-08: R2009 [17], nephelometry way with turbidimeter typed HI83749.

2.5 Wine sensory evaluation

Wine sensory analysis was carried out by a panel composed of 5 panelists (wine-makers and trained tasters). The following parameters were evaluated by the panelists: visual evaluation (colour), olfactory evaluation (aroma intensity, aroma purity), taste evaluation (spices, roundness, harmony) on a scale from one (absense of the sensation) to five (extremely intense). The scores for each property were evaluated using a statistical program called PanelCheck v1.4.2.

3 Results and Discussion

3.1 Fermentation dynamics

3.1.1. Sugar consumption and ethanol production

The results are shown in Figs. 3.and 4.

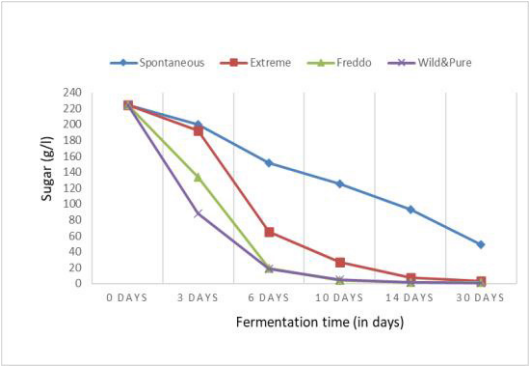

Figure 3. Reducing sugars (g/l) during alcoholic fermentation.

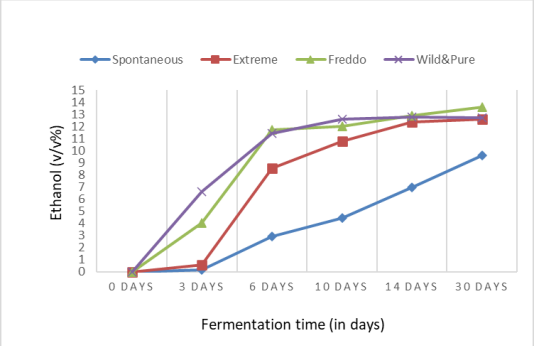

Figure 4. Ethanol production (v/v%) during alcoholic fermentation.

The soonest the **Oenoferm® Wild&Pure** began to ferment and led evenly. Like the **Oenoferm® Wild&Pure, Oenoferm® Freddo** showed a dynamic fermentation run but with higher ethanol production (7% higher alcoholic yield). **Oenoferm® X-treme** began the sugar consumption only after 3 days with a slow and sluggish start. According to the earlier studies [18], *Saccharomyces bayanus* strains are not suitable for fermentation of Kövérszőlő grape variety. The

spontaneous yeast worked with much difficulties (late start and with residual sugar) even though that the nutrient supply was assured.

3.1.2. Wine profiling and statistical analysis

The chemical compositions are shown in Table 2.

The chemical composition of wines used specied yeasts is basically different from wines made of indigenous species and the different specified yeasts also cause variation due to their different life activities.

If there is a need to reduce the negative effect of climate change, lower alcohol and higher acid yield are desirable and in this point of view **Oenoferm® Wild&Pure** shows the most favourable features. **Oenoferm® Freddo** is capable of making higher ethanol content but keeping low acid composition (8% lower then the others) caused increase in pH accompanied with dangers of non-desirable microbiologically activity.

The activity of applied specified yeasts resulted significant changes in higher alcohols such as 2,3-butanediol and 2-phenyl-ethanol content (twice amount can be occured). In the case of musts with indigenous yeasts, the presence of ppo (polyphenol oxidase enzyme) is higher and in parallel with the increase of fermentation time causes higher acetaldehyde and pyruvic acid content. **Oenoferm® Freddo** does not produce as much succinic acid content as the other specified yeast and the indigenous species.

Table 2. Results of analytical measurements.

		Spontaneous	X-treme	Freddo	Wild&Pure
Standard Parameters					
Alcohol	v/v%	9.1±0.08	13.0±0.06	13.6±0.07	12.7±0.06
Res.sugar	g/l	51.0±0.51	2.2±0.06	1.0±0.07	1.0±0.08
Glucose	g/l	18.3±0.37	nd	nd	nd
Fructose	g/l	33.0±0.23	2.0±0.01	1.0±0.01	1.0±0.01
Tartaric acid	g/l	4.1±0.04	4.0±0.37	3.8±0.07	3.8±0.09
Malic acid	g/l	1.7±0.02	1.6±0.02	1.1±0.02	1.1±0.02
Titr. acid	g/l	7.5±0.02	7.8±0.04	6.6±0.07	7.2±0.04
Volatile acid	g/l	0.6±0.01	0.39±0.07	0.36±0.01	0.36±0.04
Degradation Parameters					
Acetic acid	mg/l	197.0±0.30	100.0±0.41	245.0±0.27	200.0±0.37
Higher Alcohols/Fermentation Products					
2,3-butanediol	mg/l	174.0±0.27	255.0±0.31	347.0±0.41	267.0±0.39
2-phenyl-ethanol	mg/l	44.0±0.41	82.0±0.54	63.0±0.39	94.0±0.24
Acetaldehyde	mg/l	42.0±0.33	25.0±0.28	28.0±0.17	25.0±0.23
Pyruvic acid	mg/l	87.0±0.44	10.0±0.47	10.0±0.39	22.0±0.41
Succinic acid	mg/l	1100±0.74	1100±0.81	765.0±0.79	1100±0.83
Glycerol	g/l	6.9±0.02	7.9±0.04	6.9±0.02	8.3±0.02

In this exploratory study design, the main goal was to detect differences between three different groups (specified yeast strains) compared with the baseline settings (spontaneous). The detected differences were expressed in the form of percentages, compared to the baseline settings in each case. The pilot study data were analyzed by descriptive and percentage statistics, and the analyzed data were displayed in graphs and tables. The calculate percentage difference is usually calculated when the difference in percentage is determined between two measured variables. The measured variables from the NMR reports were extracted the data into Microsoft Excel, which was then imported into STATA v17.0 statistical software for analysis for further statistical analysis and graphical representation.

The most impressive changes were detectable in the concentration of residual sugar (X-treme: -96%, Freddo: -98%, Wild&Pure: -98%, respectively), glucose (X-treme: -100%, Freddo: -100%, Wild&Pure: -100%), fructose (X-treme: -96%, Freddo: -98%, Wild&Pure: -98%), and in the case of higher alcohols and fermentation products, such as in the concentration of 2,3-butanediol (X-treme: +47%, Freddo: -99%, Wild&Pure: +53%), 2-phenyl-ethanol (X-treme: +86%, Freddo: +43%, Wild&Pure: +114%), pyruvic acid (X-treme: -89%, Freddo: -89%, Wild&Pure: -75%) and acetaldehyde (X-treme: -40%, Freddo: -33%, Wild&Pure: -40%), respectively.

The result of the pilot study showed detectable changes. Consequently, we can infer a real effect between the effect of the three different groups compared with the baseline setting. Further investigation is needed to determine the significant level and achieve a higher level of power.

3.1.3. Degradation products with potential beneficial effects on human health

Phenolic acid contents were measured with the applied technique and the results are shown in Table 3.

Table 3. Caftaric-, gallic-, shikimic-acid and trigonelline measured values.

Phenolic acids						
		Control (must)	Sp	X-treme	Freddo	Wild&Pure
Caftaric acid	mg/l	50±5	71.0±5	85.0±5	77.0±4	87.0±2
Gallic acid	mg/l	25±2	<25	35.0±1	<25	35.0±2
Shikimic acid	mg/l	20±2	<20	<20	<20	<20
Trigonelline	mg/l	10±1	<10	13.0±1	12.0±1	12.0±1

According to the measurements, there are significant differences between applied yeast strains in producing of caftaric- and gallic acid. **Oenoferm® X-treme** and **Oenoferm® Wild&Pure** showed higher producing potential in both caftaric- and gallic acid production. In the case of caftaric acid, the spontaneous yeast flora was able to enhance the measured quantity compared to the must state. There is no difference in shikimic acid production and the difference is not significant concerning trigonelline values.

3.2 Measurements of metals

The Fig. 5 shows the copper- and iron content per treatments in the end of the fermentation (after 30 days).

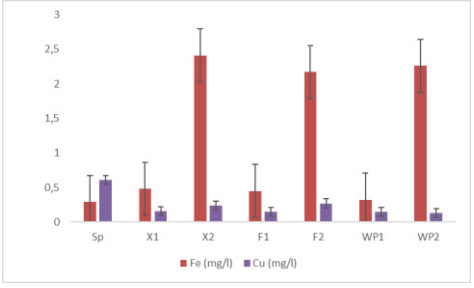

Figure 5. The measured metal values.

The copper content decreases rather than increases with the effect of added bentonite. It also presents in low concentration in the control sample (it is the spontaneous sample in this case).

There is a significant increase in iron content with the addition of the bentonite preparation used, but in none of the samples does it approach the 5 mg/l limit.

In this way, the use of bentonite during the fermentation is a safety process concerning with metal contents.

3.3 Protein stability

The ΔNTU values are shown in Table 4.

Table 4. The nephelometric values (ΔNTU<2.0 means protein-stabile state).

Samples	Sp	X1	X2	F1	F2	WP1	WP2
ΔNTU	18.0±0.1	5.70±0.1	4.30±0.05	5.20±0.2	**1.3±0.05**	6.6±0.2	**0.9±0.1**

Two treatments (F2 and WP2) with this procedure achieved a protein-stable state and a reduction of about one fifth compared to the control in the other case.

3.4 Wine sensory evaluation

The results of the sensory evaluation were assessed using 3 types of analysis: line plot, consensus spiderweb plot and eggshell plot. Although some of the information content is overlapping, each plot has its own specific characteristics and represents a unique piece of information.

Figure 6 shows the line plot analysis.

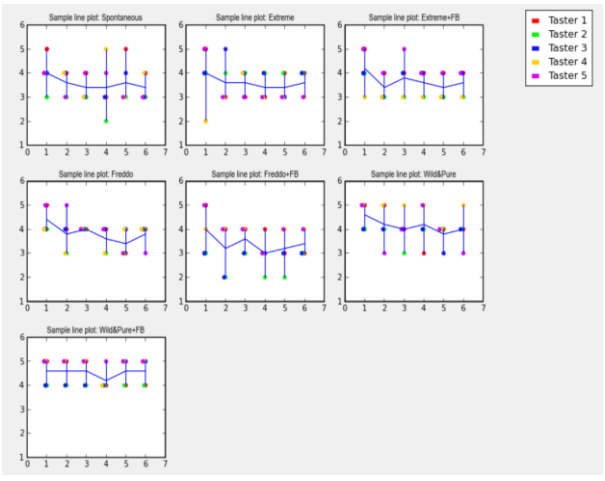

Figure 6. The sample line plots per treatments used. The attributes (horizontal axis) are colour (1), aroma intensity (2), aroma purity (3), spices (4), roundness (5), harmony (6) ; scores (vertical axis) are 1-5.

In the case of spontaneous sample, spices, roundness and colour have the widest scoring range, E1 : colour and aroma intensity, E2 : colour and aroma purity, F1: aroma intensity, F2 : colour, aroma intensity, spices, roundness, WP1 : aroma intensity, aroma purity, spices and harmony, WP2 : the same score range.

The most balance and highest scoring sample was the WP2. Comparing with WP1, the treatment of bentonite can compensate for differences and influence the sensory properties in a positive direction.

According to this plot test, F1 and F2 own the lowest scores in most attributes (aroma intensity, spices, roundness).

Figure **7** represents the the summarize of consensus datas in spiderweb plot.

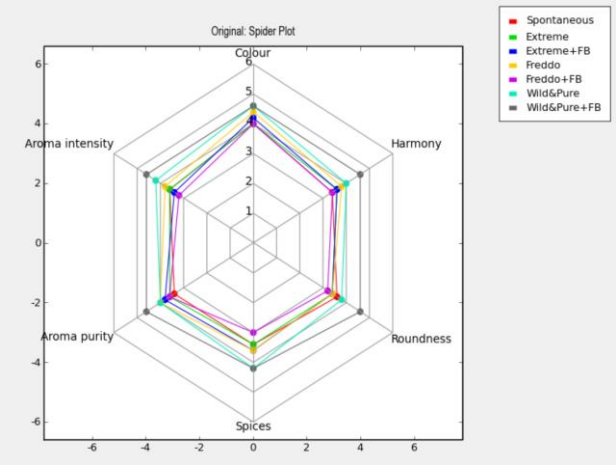

Figure 7. The consensus spiderweb plot.

WP1 and WP2 received the highest scores for all the attributes assessed, WP2 scored highest for harmony, aroma intensity, aroma purity, roundness, E2 and F2 scored poorer in aroma intensity, spiciness and harmony than the other samples.

Figure 8 views at the eggshell plot examination.

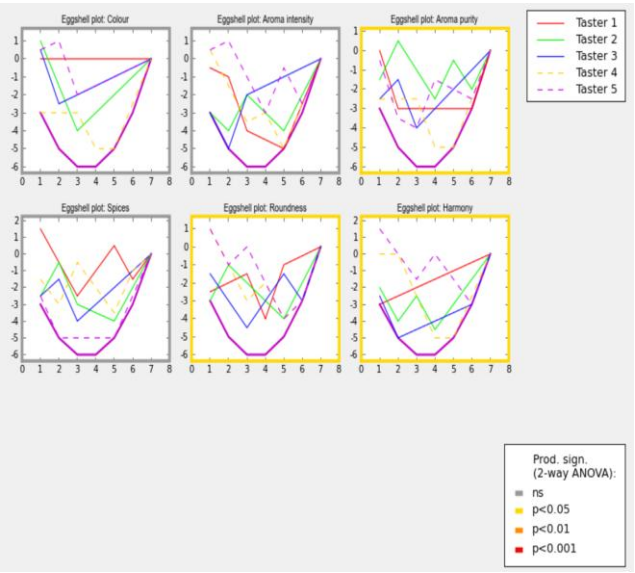

Figure 8. The eggshell overview plots according to attributes with significance level (consensus ranking (horizontal axis): 1-F2; 2-Sp; 3-X1; 4-X2; 5- F2; 6-WP1; 7-WP2; scores (vertical axis): 1-5).

With the aid of eggshell plot shows, information can be gained about how each assessor ranks all the samples tested against the consensus. There is a consensus line (consensus sample ranking) and each taster's assessment of the attributes in relation to the consensus. Plot the resulting aggregate scores against the consensus ranking, combining the scores of each rater to get a line per rater, plus a ‘‘baseline’’ formed by the consensus ranking [19]. In the case of three attributes (aroma purity, roundness and harmony) are significant difference (p<0.05) among treatments according to the tasters.

According to the results of wine sensory evaluation, there are also significant differences between treatments in terms of sensory characteristics. For the tasters, the purity of aroma, roundness and harmony are particularly important aspects in judging a given sample. The line plot can be used to select the sample of the tested items whose sensory attributes are the least shared by the judges, and whose attributes are easy to interpret and unambiguous for the consumers too. In this study the WP2 sample received the highest scores on all three tests and is the carrier of the most favourable organoleptic properties.

To sum up: Oenoferm® Wild&Pure product containing wild yeast strain seemed the best choice in the case of Tokaj Kövérszőlő dry wine because of giving lower alcohol yields and the titrable acid content stood at a good value, it started fermenting sooner and fermentation was nice and even, owning the best favourable sensory features. With the use of a wild yeast such as *Torulaspora delbrueckii*, significant quality improvements can be achieved (of course it is not enough on its own, it is necessary to add *Saccharomyces* strains to ensure complete fermentation and it is an easier way if they are together in one pack). The higher quantity of phenolic acids (e.g. caftaric acid) could be important too because of their potential health benefits (e.g., antioxidant, antibacterial, antiviral effects).

Co-fermentation of musts with bentonite can lead to early protein stabilisation, save time due to ease of use, and fermentation is clean without the formation of fermentation by-products. It can bind undesirable fermentation aromas. It results in rapid settling and no increase in heavy metal content, resulting in a cleaner, more complex flavour. Before deciding to use any technology, the most important thing is always to keep in mind the objective, what kind of wine you want to make and for which consumer audience, whether you need immediate protein stabilisation and what conditions the vintage offers. However, it is very important to be able to weigh up the dosage, because even if you increase the dosage, the purity and protein stability increase, but the treatment can be disturbing because it reduces the aromatic richness and the recognisable varietal and origin characteristics.

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