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Isolation, selection and characterisation of microorganisms with potential to form unique consortium for wine production

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Abstract

This paper focuses on a selection of microorganisms that should eventually form a Defined Consortium of Wine Microorganisms. The Consortium might serve as a sophisticated oenological product for the production of wine with attractive organoleptic features.

The Defined Consortium of Wine Microorganisms was obtained from non-saccharomyces and saccharomyces yeasts and lactic acid bacteria isolated from places where autochthonous microflora can be expected to occur (grape berries, wine lees or cellar spaces). A total of 42 microorganisms were obtained by surface smears, fallouts from the air or isolation from fermented must. All isolates were then tested and the best strains were selected on the basis of technological and phenotypic characterisation using standard microbiological techniques. The main criteria for the selection of strains were the ability to ferment and the production of organoleptically active compounds. Sulphur dioxide or ethanol tolerance, β-glucosidase activity, a taxonomic identification, the tendency to produce sulphane or the ability of lactic acid bacteria to perform malolactic fermentation were also considered. According to these results, the non-saccharomyces yeast *Starmerella bacillaris*, the saccharomyces yeast *Saccharomyces cerevisiae* and the lactic acid bacterium *Levilactobacillus brevis* were selected as part of the Defined Consortium of Wine Microorganisms.

Keywords: isolation of microorganisms; characterisation of microorganisms; yeasts; lactic acid bacteria.

1 Introduction

Must fermentation is a complex process in which different taxonomic groups of microorganisms interact (Aranda et al., 2011). In general, all these microorganisms that enter the production process, either from the grapes or from the cellar environment, can be referred to as the Consortium of Wine Microorganisms (CWM). It is a mixture of several taxa and there can be a variety of relationships between them, ranging from synergistic relationships, where the individual microorganisms help each other, to antagonistic relationships, where they compete.

In general, the main fermentative species in wine is the yeast *Saccharomyces cerevisiae*, which is the main producer of alcohol, but is in the minority at the beginning of fermentation. On the contrary, there is a high genus and species variability in the group of non-saccharomyces yeasts, which are called "starters" of alcoholic fermentation and positively modulate the aromatic complexity of the final product (Ciani et al., 2010). An equally important role is played by lactic acid bacteria, which are responsible for the conversion of malic acid into lactic acid during malolactic fermentation (Camilo et al.,2022).

The targeted selection of these microorganisms and their subsequent application to wine, in the form of a properly Defined Consortium of Wine Microorganisms (DCWM), appears to be a suitable alternative to the currently used fermentation technologies.

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The advantage of using DCWM is that the microbial composition is known and precisely defined, making it easier to control the fermentation process and predict the resulting sensory and analytical characteristics of the wine. The use of DCWM can replace the spontaneous fermentation technology, which ensures the production of unique wines, but the unknown microflora of the grapes can lead to sensory defects in the final product. The use of DCWM can also replace the fermentation of must with a pure culture of *S. cerevisiae*, which ensures a fast and reliable fermentation process with a consistent result, but the drawback of this technology might be the production of sensory and flavour uniform wines. The reason for this is the reduction in diversity of the native microbiome by a pure culture of saccharomyces yeast (Li et al.,2020). On the other hand, the resulting sensory profile is not influenced only by the yeasts but also by the grape variety, production technology or by wine training and aging.

The microbial consortia are a natural part of every vineyard. Their quantitative and qualitative composition varies and is the result of a long-term process of adaptation to the soil and climatic conditions of viticulture in different regions and localities. Anthropogenic (e.g. sprays), biotic and abiotic (precipitation, diseases, changes in temperature, humidity, UV radiation, nutrient deficiencies, etc.) aspects influence the composition of the vine microflora and the vineyards themselves (Kántor et al., 2017). In addition to the vineyard, microbial consortia are also present in the wine cellar, where they provide the natural habitat for a wide range of microorganisms, especially during the grape harvest and processing. Old stone cellars are mainly inhabited by moulds, but the presence of adapted yeasts and bacteria that prefer an environment with high humidity and low temperatures is not excluded. The microorganisms are also quantitatively present in fine yeast lees. At the end of fermentation, the yeasts and lactic acid bacteria gradually die off. These microorganisms sink to the bottom of the fermentation vessel and become the main component of the fine lees. They enrich the wine and contribute to its complexity (Fia, 2016).

Based on this knowledge, in this study, technologically important microorganisms (non-saccharomyces and saccharomyces yeasts and lactic acid bacteria) were isolated from important wine microflora habitats (vineyard, wine cellar, fine yeast lees). A detailed primary phenotypic and technological characterisation was carried out on the set of obtained yeasts, with the aim of eliminating isolates with poor technological quality parameters. Key monitored criteria included: tendency to sulphate production, yeast tolerance to free sulphur dioxide, β-glucosidase activity, fermentation rate or profile of higher alcohols and esters produced. Based on the results of these tests, representative isolates were selected to become part of the DCWM. Similar to the yeast microorganisms a detailed phenotypic, technological and taxonomic characterisation of the bacteria was carried out. Investigated parameters included: the ethanol tolerance of the bacteria and the determination of their ability to convert malic acid to lactic acid. After selection of the desired taxa, the microorganisms were cultivated and either lyophilised (yeasts) or preserved as liquid biomass (bacterium). The cultivated microorganisms formed the DCWM, which was later used in wine production. However, the selected yeasts and bacteria were not mixed together, but used separately, mainly to give each group of microorganisms enough space to develop and thus promote the uniqueness and authenticity of the wine (not an objective of this work). The present work focuses only on the method of obtaining and testing each species of microorganism.

2 Materials and methods

2.1 Culture media

WLN (WL Nutrient Agar; Oxoid): Nutrient agar for enumeration and cultivation of various types of microorganisms, mainly yeasts; bacterial growth is not excluded; helps to distinguish different types of yeasts.

WLN+T (WL Nutrient agar; Oxoid): Nutrient agar for enumeration and cultivation of yeasts; supplemented with tetracycline (T; 25 mg/l); inhibits a wide range of G+ and G- bacteria.

PCA (Plate Count Agar; HiMedia): Nutrient agar for enumeration and cultivation of yeasts and bacteria.

PCA A+ βFe (Plate Count Agar; HiMedia): Nutrient agar for the cultivation and enumeration of aerobic bacteria; supplemented with cycloheximide (actidion; 1 mg/ml) and β-phenylethyl alcohol (3 ml/l); additives inhibit eukaryotic microorganisms (actidion) and G- bacteria (β-phenylethyl alcohol).

DSM: Selective agar for the detection of acetic acid bacteria (media composition adapted from Gomes et al., 2018).

MEA (Malt Extract Agar; HiMedia): Nutrient agar for detection, isolation and enumeration of all microorganisms (yeasts; some bacteria, moulds).

MEA+T (Malt Extract Agar; HiMedia): Nutrient agar for enumeration and cultivation of yeasts; supplemented with tetracycline (T; 25 mg/l). Only eukaryotic microorganisms will grow on this medium.

MEA+T+IAA (Malt Extract Agar; HiMedia): Nutrient agar for the cultivation of non-saccharomyces yeasts

only; supplemented with tetracycline and iodoacetic acid (IAA, 0.186 g/l inhibits saccharomyces yeasts).

MRS (Lactobacillus MRS Agar; HiMedia): Nutrient agar for the identification of a wide range of lactic acid bacteria; some yeasts can grow on this medium.

MRS+A+βFe (Lactobacillus MRS Agar; HiMedia): Nutrient agar for the detection and enumeration of lactic acid bacteria; supplemented with actidion (1 mg/ml; cykloheximide) and β-phenylethyl alcohol (3 ml/l); additives inhibit eucaryotic microorganisms (actidion) and G- bacteria (β-phenylethyl alcohol).

Biggy agar (Bi.G.G.Y. Agar/Nickerson medium; HiMedia): Agar used for selective isolation, differentiation and presumptive identification of *Candida* species; in this work used for H_2S production.

2.2 Isolation of microorganisms from grape berries

The physiologically ripe grapes (variety: Welschriesling and Pálava) were harvested, pressed and the resulting unfermented must was left to ferment spontaneously at 18 °C. Before the start of the spontaneous fermentation (day 0), non-saccharomyces yeasts were isolated from the fresh must together with lactic acid bacteria. They were cultivated on a specific media in an aerobic or anaerobic environment. Saccharomyces yeasts were isolated from the fermented must with at least 10% alcohol by volume on MEA+T medium. Details of the media used, the culture conditions and the cultivation time of the microorganisms are given in Table 1.

2.3 Isolation of microorganisms from wine cellar

The following technological procedures were applied on the sampling from the wine cellar: surface smears and fallouts from the air. The methods were adapted and optimised from Kalhotka et al., 2015 (surface smears) and Říhová Ambrožová et al., 2014 (fallouts from the air). The aim of the surface smears was to isolate microorganisms from the surface of the cellar walls. A sterile cotton swab (in tube with saline solution) was used to wipe the tested area (10 × 10 cm) thoroughly and in several directions. The stick was then placed back into the tube and the initial suspension was properly diluted (10^{-1}–10^{-4}) and transferred (200 µl) to the appropriate culture media (Table 2).

The principle of the airborne fallout method was the passive transfer of microorganisms onto the surface of a solid culture medium. Two Petri dishes containing the same culture medium (PCA, MRS, MEA+T) were placed 10–30 cm apart in the wine cellar area and exposed for 45 min.

Details of the media used for the surface smears and airborne fallouts, the culture conditions and the cultivation time of the microorganisms are given in Table 2.

2.4 Isolation of microorganisms from wine lees

A sample of yeast sediment was taken from the bottom of the fermentation vessel (containing wine) into a sterile plastic tube via a sampling tap. The lees sample was centrifuged (10,000 rpm, 5 min, 10 °C) and diluted accordingly 10^{-1}–10^{-6}. Then 200 µl of the sample was pipet-

Table 1 *Table of used solid culture media and cultivation conditions*

Solid culture medium	Expected growth of MO	Cultivation temperature	Type of cultivation depending on oxygen	Cultivation time
MEA+T	S, NS	30 °C	aerobic	3 days
MEA+T+IAA	NS			
WLN+T	S, NS			
MRS A+ βFe	B (G+)		aerobic and anaerobic	5–7 days

S – saccharomyces yeasts, NS – non-saccharomyces yeasts, B – lactic acid bacteria, G+ –grampositive bacteria; MO – microorganism

Table 2 *Table of used solid culture media and cultivation conditions*

Solid culture medium	Expected growth of MO	Cultivation temperature	Type of cultivation depending on oxygen	Cultivation time
PCA	S, NS, B	30 °C	aerobic	3 days
MEA	S, NS, partly B			
MEA+T	S, NS			
MEA+T+IAA	NS			
MRS	B (G+; G-)		aerobic and anaerobic	5–7 days

S – saccharomyces yeasts, NS – non-saccharomyces yeasts, B – lactic acid bacteria, G+ –grampositive bacteria, G- – gramnegative bacteria; MO – microorganism

ted onto the solid culture medium (PCA, MEA, MEA+T, MEA+T+IAA, WLN, MRS, MRS A+βFe). The culture conditions of the prepared Petri dishes were identical to those described in section 2.2 and 2.3.

2.5 Yeast characterisation

2.5.1 Nanovinification

24 ml of chemically-defined synthetic grape must (3 g/l yeast extract; 6 g/l proteose peptone; 3 g/l tartaric acid, 5 g/l malic acid; 0.5 g/l citric acid monohydrate; 0.00712 g/l $ZnSO_4.7H_2O$) with 260 g/l fermentable sugars (glucose and fructose) was inoculated with a single colony of non-saccharomyces target microorganism from Petri dish. The fermentation was carried out at 18 °C and it took 35 days. Each test was carried out in triplicate. Nanovinifications were aerated by opening the tube in a laminar box once per week.

After nanovinification, the content of the desired analytes (ethanol, ethyl acetate, acetic acid) was determined by gas (GC; section 2.5.3) and liquid chromatography (HPLC; section 2.5.3). If the results of liquid and gas chromatography showed higher than tolerable concentrations of acetic acid (>1.5 g/l) or ethyl acetate (>150 mg/), the yeasts were excluded from the group of the tested isolates. These isolates were not suitable for the establishment of vinifications in larger volumes (microvinifcation).

2.5.2 Microvinification

The day before the microvinification the tested yeast strains were inoculated into 25 ml of YPD (20 g/l glucose; 20 g/l proteose peptone; 10 g/l yeast extract) medium in Erlenmayer flask and cultured for 24 h at 30 °C on orbital shaker (130 rpm). After 24 h of culturing, the number of cells in the culture was determined using a Bürker chamber. After that a chemically-defined synthetic grape must in Erlenmayer flask (150 ml, the same composition as in section 2.5.1) with 260 g/l fermentable sugars was inoculated with 10^6 cells/ml of target microorganism (saccharomyces and selected non-saccharomyces yeasts). The Erlenmeyer flask was fitted with a fermentation stopper with 2 ml of 75% glycerol to prevent possible distortion due to water evaporation. The fermentation was carried out at 18 °C and the weight loss was monitored gravimetrically. Each test was carried out in triplicate. The results are presented as an arithmetic mean with relative standard deviations. The obtained data were used to calculate the required variables representing the technological properties of yeasts (ethanol production rate, maximum ethanol production, fermentation rate or lag phase length).

2.5.3 GC, HPLC analysis

After the nano and microvinification, the synthetic medium was used for chemical and volatile profile analysis. The residual sugars, alcohol content and acetic acid were determined by High Performance Liquid Chromatography (HPLC) on Agilent 1260 Infinity using Hi-Plex H (300 × 7.7 mm) column with RID detector, VWR 260 nm. The software OpenLab CDS was used for chromatogram analysis. The higher alcohols and ester content was measured by Gas Chromatography GC-450 Bruker using Rxi 624 Sil MS (30 m × 0.25 mm × 1.4 µm) column with FID detector. Based on these results the esters and higher alcohols score was calculated. This score expresses the sensory activity of total measured esters/higher alcohols by multiplication of their measured concentration by sensory threshold concentration. Unlike the real concentration, it demonstrates the actual contribution of the substance to sensory profile. Based on these data, the sensory profile of the yeasts was classified as ester (floral or fruit-like) or neutral. Each analysis was carried out in triplicate from three independent tests.

2.5.4 H_2S production

The determination of sulphate production was carried out on specific BiGGY agar (Oxoid, method adapted and optimized from Castellucci, 2012). 8 µl of fresh yeast biomass suspension at a concentration of 10^8 cells/ml was pipetted onto the top of the solid culture media. The BiGGY agar contained bismuth sulphite, which reacted with the hydrogen sulphide produced by the yeasts to form a black precipitate of bismuth sulphide. The evaluation was performed after 48 h of static cultivation at 18 °C. The intensity of colony colour corresponded to the amount of sulphate produced.

2.5.5 Free sulphur dioxide tolerance

An adequate (0; 0.3; 0.7; 1.0; 1.3; 2.0; 2.7; 3.3 ml) amount of $K_2S_2O_5$ stock solution (2.6 g/l) was added to the YNB medium (6.75 g/l YNB; 20 g/l dextrose; pH 3.5) so that the resulting concentration of free SO_2 in the 50 ml of stock solution (YNB+SO_2) was 0; 9.2; 18.5; 27.7; 32.8; 48.8; 64.7; 82 mg/l (the method of free SO_2 quantification adapted from OIV, 2018). Tolerance was determined only in case of non-saccharomyces yeasts, which are more sensitive to the presence of sulphur dioxide. The prepared SO_2 stock solutions (YNB+SO_2) were inoculated with 10^6 cells/ml (quantified by microscopic cell counting) of non-saccharomyces microorganisms and transferred (200 µl) onto a microtiter plate in technical triplicate. Optical density measurements were performed after 48 h of culturing (25 °C) at a wavelength of 600 nm. At the end of the experiment, the experimental data were processed in the R 3.6.3 environment (Dalgaard, 2010). OD_{600} values measured after 48 h of incubation were

interleaved with a four-parameter type 1 Weibull model using the 'drc' library (Ritz and Strebig, 2016), with the input data transformed using the Box-Cox method (Box and Cox, 1964). Outlying points were identified based on the distance of their deviations from the model ($\chi 2$ test; $\alpha = 0.95$). The isolate tolerance, expressed and recalculated as the concentration of free sulphur dioxide at which the growth response is halved, was then calculated from the model.

2.5.6 β-Glucosidase activity

β-Glucosidase activity was determined spectrophotometrically as the amount of released yellow-coloured product p-NP (4-nitrophenol) from *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG, substrate) at 405 nm (Strahsburger et al., 2017).

2.5.7 Taxonomic identification of yeasts

Yeast strains were identified using ITS fragments amplified with primer pair ITS4/ITS5 (TCCTCCGCTTATTGATATGC; GGAAGTAAAAGTCGTAACAAGG). For the reaction, Q5 DNA polymerase (NEB) was used according to the manufacturer's protocol and whole cells boiled in 20 mM NaOH were used as a template. Amplicons were checked using horizontal electrophoresis (1.2% agarose, 30 min @ 10 V/cm), purified using QIAquick PCR Purification Kit (Qiagen) and Sanger-sequenced using the ITS1 primer (TCCGTAGGTGAACCTGCGG). The sequence was analysed using the UNITE database online tool (Nilsson et al., 2019).

2.6 Lactic acid bacteria (LAB) characterisation

2.6.1 Ethanol tolerance

The liquid MRS medium was supplemented with ethanol so its final concentration in the medium was 2; 4; 6; 8; 10 and 12.5% vol. The prepared stock solutions were inoculated with lactic acid bacteria (10^6 cells/ml) and transferred (200 µl) onto a microtiter plate in technical triplicate. Optical density measurements were performed after 72 h of culturing (25 °C), at a wavelength of 600 nm. The interpretation of the data was similar to that described in section 2.5.5. The mathematical model was then used to calculate the isolate tolerance, expressed as the concentration of ethanol at which the growth response would be halved.

2.6.2 Taxonomic identification of LAB

16S rRNA sequencing was used for taxonomic identification. Cells boiled in 20 mM NaOH were used as templates. Polymerase Q5 (New England Biolabs) and primers 27F and 1492R were used for amplification. The rest of the taxonomic identification procedure was analogous to that described in section 2.5.7. 337F was used as the sequencing primer. Sequences were analysed using the EzBioCloud environment (Yoon et al.,2017).

2.6.3 Testing the ability of LAB to convert malic acid to lactic acid

150 ml of chemically defined synthetic grape must (same as in section 2.5.1) with 210 g/l fermentable sugars (glucose and fructose) and 5 g/l of malic acid was inoculated with 10^6 cells/ml of saccharomyces yeast (EPS 1096) and lactic acid bacteria. LAB were applied to the microvinification at 0%, 2% and 4% alcohol by volume. The fermentation was carried out at 20 °C and its progress was monitored gravimetrically. Once a week a sample of the synthetic medium was taken for microbiological analysis of the viability of lactic acid bacteria. This was determined by diluting (10^{-8}–10^{-11}) 1 ml of the homogeneous sample and transferring (200 µl) into the MRS A+βFe culture medium. The Petri dishes were cultured in an anaerobic environment at 30 °C for 5–7 days. The bacterial growth was then assessed as the number of colony forming units in 1 ml of sample (CFU/ml). The same sample was used to assess the conversion of malic acid to lactic acid by liquid chromatography (HPLC, section 2.5.3). Each test was performed in triplicate. The results are presented as arithmetic means with relative standard deviations. From the obtained experimental data, the dependence of the decrease in cells on time and the dependence of the decrease in malic acid and the increase in lactic acid on the duration of fermentation were determined.

3 Results and discussion

3.1 Isolation of microorganisms from grape berries, wine cellar and wine lees

A total of 42 microorganisms were isolated from these matrices, of which 7 came from the wine cellar, 9 from fine wine lees and 26 from grapes. From these data it can be concluded that the best matrix for isolation is the grape, where the greatest number of variable isolates (saccharomyces, non-saccharomyces yeasts and lactic acid bacteria) was obtained. The qualitative representation of the individual microorganisms was as follows: 5 saccharomyces yeasts, 28 non-saccharomyces yeasts and 9 lactic acid bacteria.

3.2 Yeast characterisation

3.2.1 Nanovinification

This test eliminated 1 isolate with high acetic acid production from 28 non-saccharomyces yeasts. Yeasts with no ethanol production were also identified as non-compliant. 10 isolated non-saccharomyces yeasts were found to be non-fermentable (ethanol production was 0% vol.). The other 7 isolates produced relatively high ethanol concentrations compared to the other yeasts, but also

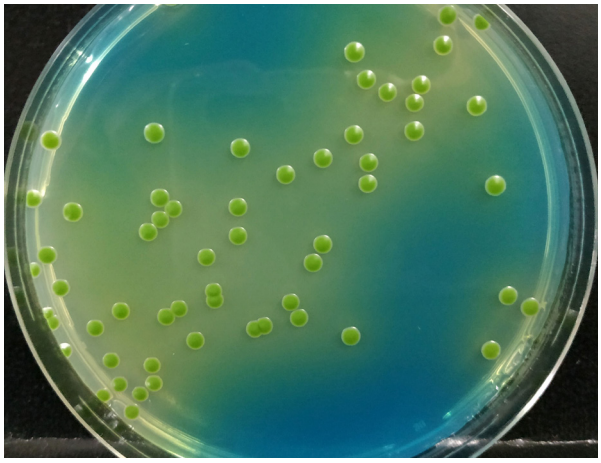

Figure 1 *Yeasts on solid culture media WLN+T*

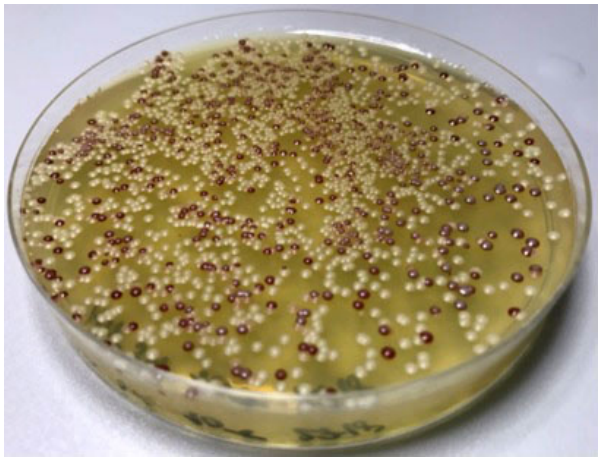

Figure 2 *Mixed culture of microorganisms on MEA medium*

non-tolerable (260–460 mg/l) concentration of ethyl acetate. A further 3 yeasts showed an excessive agglutination rate, which would have prevented an easy scale-up process. 4 yeasts showed poor growth on culture media. On this basis, 3 isolates – EPS 1224, 1225 and 1226, were selected from the wide range of isolated non-saccharomyces yeasts for further characterisation tests.

3.2.2 Microvinification

Among the non-saccharomyces yeasts, EPS isolates 1224 and 1225 had a sufficient fermentative capacity, while EPS 1226 had an above-average fermentative capacity. Among other things, this tested isolate showed a three times higher glycerol production (up to 6 g/l) compared to the other non-saccharomyces yeasts (1.3–4.6 g/l). Excellent fermentation ability was also observed for the saccharomyces isolates. The only exception was one isolate (EPS N002), which showed no signs of growth in the used synthetic medium. For this reason, it was considered as "technologically unsuitable" and was excluded from further characterisation tests. From the presented data (Table 3) it can be concluded that none of the isolates produced ethyl acetate in excess and acetic acid production was at the sensory threshold.

3.2.3 H_2S production

The determination of this parameter by the semi-quantitative screening method was only informative and did not eliminate isolates. In the group of tested yeasts were 2 with low (EPS 1223; 1226), 1 with medium (EPS 1225) and 4 with high sulphate production (EPS N003; 1096; 1099; 1224).

3.2.4 Free sulphur dioxide tolerance

Knowledge of the tolerance of non-saccharomyces yeasts to free sulphur dioxide (SO_2) is essential in order to apply the correct dose of sulphur to the grapes during the maceration process. The concentrations indicated can be considered as the minimum amount of sulphur dioxide at which the metabolic activity of the yeasts is reduced. The results of the tolerances of the isolates tested are shown in Table 3. It is clear from the data that the non-saccharomyces yeasts, represented by isolates EPS 1225 and EPS 1226, tolerate approximately half the concentrations of free sulphur dioxide compared with isolate EPS 1224. Although the tolerance characteristic of these yeasts is significantly lower, their application is also suitable for musts that have been treated with a dose of SO_2 during the grape processing technology. However, care must be taken to ensure that the used concentration of free SO_2 in the must does not exceed the concentrations given in Table 3.

3.2.5 β-Glucosidase activity

The enzyme β-glucosidase facilitates the volatilisation of glycosidically bound terpenes, which become organoleptically active when released from the bond. The use of yeasts with this enzymatic equipment appears to be an effective way of making the aromatic profile of the resulting wine more attractive. The set of saccharomyces yeasts tested includes isolates with high (EPSN 001, EPSN 003, EPS 1099) and very high (EPS 1096) enzymatic activity. In the case of the non-saccharomyces yeasts, a significant release of sensory active compounds of terpenoid nature is unlikely, as they possess only low or medium levels of activity of this enzyme.

3.2.6 Taxonomic identification of yeasts

Taxonomic identification of the selected isolates was performed by sequencing ITS regions in the genome of eukaryotic microorganisms. Only those yeasts were sequenced whose technological attractiveness had been demonstrated by characterisation tests (EPS 1096, 1224, 1225, 1226). As expected, the species and genus analy-

sis of the saccharomyces yeast confirmed that it was an isolate of *Saccharomyces cerevisiae*. A high level of generic diversity was found in the non-saccharomyces group of microorganisms. The technologically important yeast *Starmerella bacillaris*, characterised by high glycerol production, was also isolated (Englezos et al., 2017). The results of the taxonomic characterisation are presented in Table 3. Information on the set of selected eukaryotic microorganisms is complemented by microscopic photographs (Figure 3).

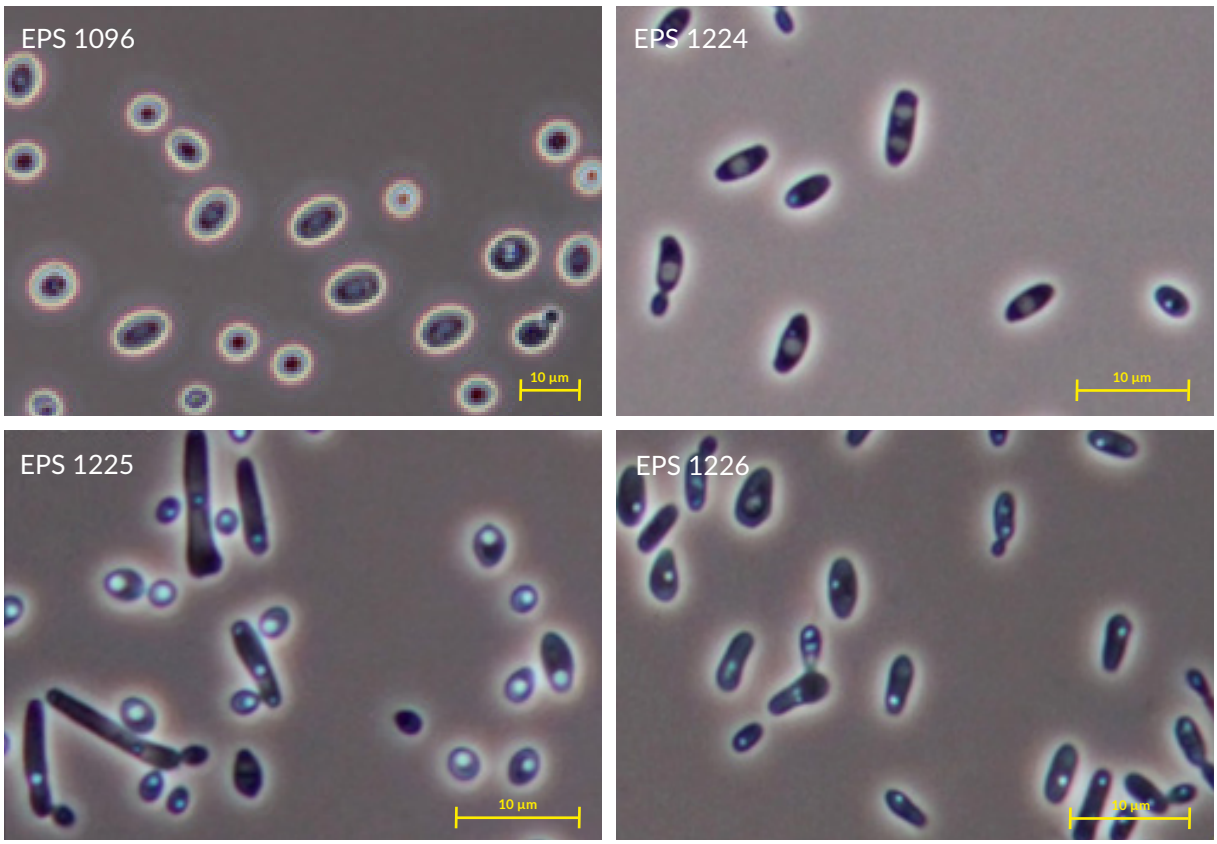

Figure 3 *Microscopic features of the selected*

Table 3 *Summary of phenotypic and taxonomic characterisations of eukaryotic microorganisms (saccharomyces and non-sacharomyces yeasts)*

Type of MO	Tested isolate	c_{EtOH} (% vol.)	$c_{ethyl\ acetate}$ (mg/l)	$c_{acetic\ acid}$ (g/l)	Sensory expression	H_2S production	β-glucosidase activity	IC50 (SO_2) (mg/l)	max.EtOH (% vol.)	Taxonomical identification
S	EPS 1223	14.5	18.0		neutral	low	high	NA	15.7±0.2	NA
	EPSN 002	Technologically unsuitable isolate								
	EPSN 003	15.1	12.2	1.2	ester-like	high	high	NA	13.5±0.1	NA
	EPS 1096	15.1	14.6	1.0	neutral	high	very high	NA	15.0±0.0	*Saccharomyces cerevisiae*
	EPS 1099	15.1	8.3	1.1	neutral	high	high	NA	14.5±0.1	NA
NS	EPS 1224	2.0	< 1.5	1.2	neutral	high	medium	31.9±0.8	3.9±0.1	*Ogataea boidinii*
	EPS 1225	2.4	< 1.5	0.0	neutral	medium	low	16.3±0.8	3.5±0.2	*Yamadazyma friedrichii*
	EPS 1226	7.1	< 1.5	0.8	neutral	low	medium	15.6±1.1	8.1±0.2	*Starmerella bacillaris*

S – saccharomyces yeasts; NS – non-saccharomyces yeasts; MO – microorganism; c – concentration;
IC50 – concentration of sulphur dioxide at which the growth response is halved;
c_{EtOH} – actual alcohol concentration produced during microvinification on synthetic medium;
max. EtOH – maximum theoretical alcohol production on synthetic medium; NA – data not analysed

3.3 Lactic acid bacteria characterisation

3.3.1 Ethanol tolerance

Lactic acid bacteria were selected on the basis of their tolerance to ethanol. In general, the higher the tolerance to ethanol, the more suitable the bacterium is for carrying out malolactic fermentation (MLF). This is mainly because lactic acid bacteria are most often inoculated into wine after the main alcoholic fermentation (sequential inoculation), at a time when the amount of alcohol produced is already at or above 10% ethanol by volume. Inoculation at the beginning of fermentation (co-inoculation) is not excluded, but care must be taken to ensure that the lactic acid bacteria are able to carry out malolactic fermentation before it loses their viability (Costello et al., 2015). From the 9 originally isolated bacteria, 2 were discarded due to poor growth characteristics in the pre-culture medium. The test results showed that 3 bacteria with higher (>10% vol.) as well as bacteria with a lower tolerance (<10% vol.) to ethanol were present in the group of tested lactic acid bacteria (Table 4). From a technological point of view, the bacteria with higher tolerance are more attractive, especially because their application is also suitable for wines with a high alcohol content.

3.3.2 Lactic acid bacteria identification

Taxonomic identification is crucial in the selection of lactic acid bacteria, especially since the OIV Codex (2021) states that only the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Oenococcus* can be used in wine. If the speciation of the tested bacteria showed a genus other than the permitted ones, the bacteria were not used in the production of wine. The lactic acid bacteria EPS 1227 and EPS 1408 were selected for identification according to the results of ethanol tolerance (reported in section 3.3.1), growth characteristics on solid culture media (not reported) and in the pre-culture media (not reported). Both isolates belonged to the lactobacilli group, which allows their use in oenology. The lactic acid bacterium EPS 1227 was classified as *Lacticaseibacillus paracasei*, while the isolate EPS 1408 was classified as *Levilactobacillus brevis*. The second one is considered to be one of the most commonly used lactic acid bacteria in the production of fermented beverages, including wine. Information on the set of selected prokaryotic microorganisms is complemented by their microscopic photographs (Figure 4).

Table 4 *Tolerance of lactic acid bacteria to ethanol*

Tested isolate	IC50 (EtOH) (% vol.)
EPSN 012	11.7 ± 0.6
EPS 1227	12.2 ± 1.2
EPSN 014	11.3 ± 0.1
EPSN 015	7.7 ± 2.6
EPS 1408	9.3 ± 0.2
EPSN 046	8.7 ± 0.2

3.3.3 Testing the ability of LAB to convert malic acid to lactic acid

To test the ability to convert malate to lactate, isolate EPS 1408 was selected on the basis of taxonomic identification. The degradation of malate, the evolution of alcohol, lactic acid content and the decrease in bacterial population are shown in Table 5.

It is clear from these data that the selected lactic acid bacterium is partially capable of converting malic acid to lactic acid. The ability to convert malic acid to lactic acid is only present when the bacterium is used in the co-inoculation strategy (together with the cultured yeast) at 0% alcohol. The bacterium was able to produce 1 g of lactic acid during the 21 days of fermentation, which corresponds approximately to the balance reported in the literature, according to which the degradation of 1 g of malic acid produces 0.67 g of lactic acid (Cavaglia et al.,

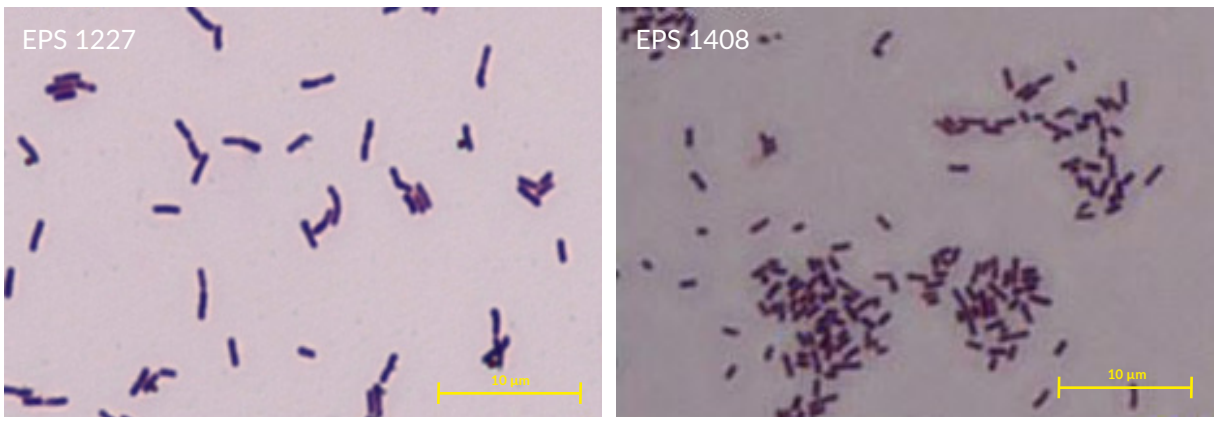

Figure 4 *Microscopic photographs of lactic acid bacteria EPS 1227 and EPS 1408*

Table 5 *Results of simulated malolactic fermentation with malolactic bacterium EPS 1408*

	Fermentation day	Alcohol content (% vol.)	CFU (cells/ml)	Malic acid (g/l)	Lactic acid (g/l)
LAB inoculation at 0% alcohol by volume	0.	0.0±0.0	1.0E+06	4.3±0,0	0.0±0.0
	8.	6.8±1.2	9.8E+05	3.6±0,0	0.6±0.0
	14.	10.1±1.2	5.6E+05	3.5±0,0	0.9±0.0
	21.	11.4±0.4	1.3E+05	3.0±0,1	1.0± 0.0
	28.	12.4±0.1	9.0E+04	3.0±0,1	1.0±0.1
LAB inoculation at 2% alcohol by volume	0.	2.0±0.0	1.0E+06	4.3±0,0	0.0±0.0
	9.	9.2±0.5	5.6E+04	4.1±0,0	0.1±0.0
	16.	11.1±0.5	3.4E+04	4.1±0,0	0.2±0.0
	23.	11.9±0.3	2.3E+04	3.7±0,4	0.2±0.0
	30.	12.4±0.0	9.1E+03	4.0±0,1	0.1±0.0
LAB inoculation at 4% alcohol by volume	0.	4.0±0.0	1.0E+06	4.3±0,0	0.0±0.0
	7.	9.1±0.0	1.2E+05	4.2±0,1	0.1±0.0
	14.	11.1±0.2	1.1E+05	4.1±0,0	0.2±0.0
	21.	12.1±0.1	1.1E+04	4.1±0,1	0.2±0.0
	28.	12.4±0.2	4.0E+03	3.9±0,1	0.2±0.0

LAB – lactic acid bacteria, CFU – Colony Forming Unit

2022). The isolate tested is not able to successfully carry out malolactic fermentation of musts with a malic acid content above 1.5 g/l due to the rapidly increasing alcohol content. The use of this bacterium is therefore limited to musts with a low malic acid content and a higher pH (preferably 3.5). Analysis of CFU and minimum lactic acid production has shown that it is inappropriate to use the bacterium in 1/3 of the fermentation (at 2% and 4% alcohol by volume), because of rapid cell death which occurs within the first 7 days of inoculation (cell number decreases by at least 1 order).

3.4 Final selection of microorganisms

Taking into account the results of the primary and taxonomic characterisation, the saccharomyces yeast EPS 1096, the non-saccharomyces yeast EPS 1226 and the lactic acid bacterium EPS 1408 were selected for the Defined Consortium of Wine Microorganisms. The final step after the selection of the microorganisms was a large-scale cultivation and subsequent lyophilisation (not the aim of this work).

4 Conclusion

In this work, methods of isolation and characterisation of important microorganisms from different oenological matrices was developed. It was shown that the matrix with the most diversified microbial composition is the grape. Up to 62% of the total obtained microorganisms came from this matrix. The remaining microorganisms were recovered from a wine cellar (17%) or from fine yeast lees (21%). Methods for characterising individual taxa were also optimized, with emphasis on the ability to conduct alcohol or malolactic fermentation without sensory defects or other deviations. On the basis of the obtained data, 3 types of microorganisms were selected to eventually form the Defined Consortium of Wine Microorganisms. The saccharomyces yeast *Saccharomyces cerevisiae* is responsible for the smooth running of the main alcoholic fermentation, while the non-saccharomyces yeast *Starmerella bacillaris* might improve the aromatic complexity of the final product. In addition, the use of this yeast positively modulates the strength and fullness of the wine's flavour, thanks to its increased production of glycerol. The last taxonomic group of the Consortium is the lactic acid bacteria, represented by *Levilactobacillus brevis*, which is responsible for malolactic fermentation. The use of a Defined Consortium of Wine Microorganisms with a precise composition is intended to improve the quality of the wine in terms of the sensory and analytical parameters of the resulting product.

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