NATIVE YEAST FROM DISTINCT ORGANS OF GRAPEVINES ESTABLISHED IN QUERETARO, MEXICO, AND THEIR POTENTIAL OENOLOGICAL UTILIZATION

LEVEDURAS NATIVAS DE DIFERENTES ORGÃOS DE VIDEIRAS INSTALADAS EM QUERETARO, MÉXICO, E A SUA POTENCIAL UTILIZAÇÃO ENOLÓGICA

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SUMMARY

The aim of this study was to isolate, identify and determine the oenological potential of yeasts present in *Vitis vinifera* organs of grapevines established in Queretaro State, Mexico. The yeast distribution was influenced by the organ and the sampling season, and the yeast populations ranged from 0.8 and 5.5 Log CFU/g. A total of 93 yeasts were isolated, identified by RFLP and confirmed by sequencing of the ITS region, prevailing *Aureobasidium* cf. *melanogenum* and Basidiomycota yeast. The identified species with previously reported oenological potential were: *Pichia* cf. *kluyveri* and *Clavispora* cf.*opuntiae*. Remarkably, *P.* cf. *kluyveri* 3.1HM showed killer phenotype and was the most tolerant to sulfur dioxide, and survived 72 h after its inoculation in 'Tempranillo' must. *C.* cf. *opuntiae* 5.7HM showed β-glucosidase activity, the highest tolerance to 5 % ethanol and 25 °Brix (sugar levels). On the contrary, *Rhodotorula* isolates were not tolerant to stress conditions, and *R. mucilaginosa* 8HM did not grow under must conditions. Mixed fermentation using *H. uvarum* NB108/*S. cerevisiae* N05 resulted in the highest volatile acidity (0.45 g/L acetic acid), while no differences for total acidity, alcohol strength, residual sugars and total SO_2 were found between the mixed fermentations treatments. This study provides an insight into the yeast diversity present in grapevines established in Queretaro, Mexico, and the oenological potential of. *P.* cf. *kluyveri* 3.1HM.

RESUMO

O objetivo deste trabalho foi isolar, identificar e determinar o potencial enológico de leveduras presentes em órgãos de videiras da espécie *Vitis vinifera* instaladas no estado de Queretaro, México. A distribuição das leveduras foi influenciada pelo orgão e pela época de amostragem, e as populações de leveduras variaram entre 0,8 e 5,5 Log UFC/g. Nas 93 leveduras isoladas, identificadas por RFLP e confirmadas por sequenciação da região ITS, observou-se uma predominância de *Aureobasidium* cf. *melanogenum e* Basidiomycota. As espécies identificadas com potencial enológico previamente relatado foram: *Pichia* cf. *kluyveri* e *Clavispora* cf. *opuntiae*. Importa salientar que *P.* cf. *kluyveri* 3.1HM revelou fenótipo *killer* e maior tolerância ao dióxido de enxofre, tendo sobrevivido 72 h após inoculação em mosto de uvas da casta 'Tempranillo'. *C.* cf. *opuntiae* 5.7HM apresentou atividade β-glucosidase, bem como a maior tolerância a 5 % de etanol e a 25 °Brix (teor de açúcares). Em oposição, os isolados de *Rhodotorula* não mostraram tolerância às condições de stress, e *R. mucilaginosa* 8HM não cresceu nas condições do mosto. As fermentações mistas, utilizando *H. uvarum* NB108/*S. cerevisiae* N05, originaram a acidez volátil mais elevada (0.45 g ácido acético/L); não foram detetadas diferenças na acidez total, no título alcoométrico, no teor de açúcares e no teor total de dióxido de enxofre entre as modalidades de fermentação mista. Este estudo permite compreender a diversidade de leveduras presentes em videiras instaladas em Queretaro, México, e o potencial enológico de *P.* cf. *kluyveri* 3.1HM.

Key words: *Vitis vinifera* organs, yeast distribution, non-*Saccharomyces*, stress conditions, rDNA sequencing.
Palavras-chave: Orgãos de *Vitis vinifera*, distribuição de leveduras, não-*Saccharomyces*, condições de stress, sequenciação de rDNA.

INTRODUCTION

Wine is the beverage resulting from the alcoholic fermentation of grape must and it is carried out by a sequential action of yeasts (OIV, 2019a). During the first steps of fermentation the so-called non-*Saccharomyces* (n-S) dominate (Jolly *et al*., 2014). Due to their low tolerance to ethanol, diversity of this group gradually decreases as the fermentation advances and *Saccharomyces* species become dominant and complete the process (Varela and Borneman, 2017).

During their residency on must fermentation, native yeast especially n-S, produce certain secondary metabolites, such as glycerol, esters, higher alcohols, medium chain fatty acids, among others, which positively affect the wine quality, conferring higher complexity to wine (Varela, 2016). Moreover, the inoculation of selected native strains to perform must fermentation is blooming, since the strains may be better adapted to the environmental conditions and could also potentiate the tipicity of wines (Callejón *et al*., 2010; Capozzi *et al.,* 2015; Padilla *et al.*, 2016a; Miranda *et al*., 2017).

The isolation and selection of oenological yeasts is generally performed from mature grapes and must in fermentation (Belda *et al.,* 2016; Liu *et al.*, 2016; Padilla *et al.,* 2016b). Besides, in vineyards, yeasts could be found on stems, leaves, and on the surface of berries, mainly in stomata, lenticels and sites where microfissures are present (Bernardi, 2013).This yeast diversity was rarely considered to be studied (Garijo *et al*., 2011), on the belief that strains with oenological potential could be mainly found in wineries.

Yeast genera present in grapevines may be classified into three main groups: a) oxidative yeasts without any oenological interest (*Cryptococcus*, *Sporobolomyces*, *Aureobasidium*); b) mildly fermentative yeasts (*Hanseniaspora*, *Pichia*, *Metschnikowia*); c) fermentative yeasts (*Saccharomyces*, *Torulaspora*, *Zygosaccharomyces*, *Lachancea*) (Barata *et al*., 2012).

The recent blooming of the wine industry in Queretaro, Mexico, demands to explore quite new yeast diversity difficult to have in other winemaking areas, where the age of the vineyards and their proximity to wineries has led to a more selected microbiota. Research concerning the selection of native oenological *Saccharomyces* (Miranda-Castilleja *et al*., 2015) and n-S (Ortiz-Barrera *et al*., 2015) yeasts has been performed in this region. Nevertheless, there is a lack of information about the diversity and distribution of native yeasts in the vineyard and their possible use in winemaking in Mexico, which could lead to the possibility of finding interesting and original strains with enological benefits. Therefore, the aim of this study was to isolate, identify and determine the oenological potential of yeasts present in different organs of grapevines in the region.

MATERIAL AND METHODS

Sampling and yeast strains

Samples were collected from six-year-old grapevines (*Vitis vinifera* L. cv. 'Tempranillo' grafted on SO4 rootstock), established in Los Cues, Queretaro, Mexico. The parts of the vines sampled according to their vegetative cycle (Table I) were randomly collected along three lines of the vineyard (triplicate), put into plastic bags (10 x 20 cm), transported inside a cooler and immediately processed in the laboratory. The commercial strain *S. cerevisiae* K1-V1116 (Lalvin, Ontario, Canada) and the native selected strain *S. cerevisiae* N05 (Miranda-Castilleja *et al*., 2015) were used. Besides, the sensitive to killer phenotype reference strain AH22 (ATCC38626) as well as two n-*S Hanseniaspora uvarum* (NB27 and NB108) controls (Ortiz-Barrera *et al*., 2015) were also used.

Table I

Sampling month and grapevine parts sampled from a 'Tempranillo' vineyard established in Los Cues, Mexico

Mês de amostragem e partes da videira amostradas numa vinha da casta 'Tempranillo' instalada em Los Cues, México

Grapevine part	Sampling month					
	May	June	July	August	September	October
Inflorescence	X					
Fruit set		X				
Veraison fruit			X			
Ripe fruit				X		
Apical leaf		X				
Medium leaf		X	X	X	X	X
Basal leaf		X	X	X	X	X
Latent Bud						X

Isolation of yeasts

For each triplicate of sample collected, a total of 50 berries of each physiological state, 3 g of inflorescences or buds and 5 g of leaves were placed separately in plastic bags containing 50 mL of peptone diluent (0.1 % peptone of casein). Berries

were carefully and manually homogenized for 3 min. The other samples were homogenized separately in a Stomacher at medium velocity during 1 min. Suspensions were placed in 50 mL conical tubes and centrifuged at 1790 g for10 min in order to obtain the pellet containing the microorganisms.

Three serial dilutions 1:10 were made and 100 μL were spread plated in nutrient yeast dextrose agar (NYDA, 8 g/L nutritive broth, 5 g/L yeast extract, 10 g/L dextrose and 15 g/L agar) supplemented with rose Bengal (100 mg/L) and chloramphenicol (100 mg/L). Plates were incubated at 28 °C for 48 h, counts were made, and contrasting colony morphologies were isolated in NYDA.

Identification of yeasts species

For the DNA extraction, a loop of each isolate colony collected from the agar was resuspended in 300 μL of TNES (Tris-HCl 200 mM pH 8.5, NaCl 250 mM, EDTA 25 mM, SDS 0.5%) and, from this step, the methodology described by Crespo-Sempere *et al.* (2013) was followed.

For PCR, 2 μL of DNA from each isolate were added into 48 μL of reaction mix containing: 10 μM ITS1 (5' TCCGTAGGTGAACCTGCGG 3'), 10 μM ITS4 (5' TCCTCCGCTTATTGATATGC3'), 2mM of dNTPs, buffer 10X, taq polymerase and PCR-grade water. The PCR reaction was carried out in a Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific, CA, USA) following the next conditions: 95 °C for 2 min; 35 cycles at 94 °C for 15 s, 55 °C for 30 s; 72 °C for 30 s; 72 °C for 5 min. The PCR products were separated by electrophoresis in 1.3 % agarose gel using 100 V for 30 min. Then, PCR products were separated in three tubes and restriction enzymes *Hha*, *HaeIII* and *HinfI* were independently added; each reaction was incubated at 37 °C for 3 h. The obtained fragments after the enzymatic digestion were separated in a 3 % agarose gel. The size of the restriction fragments was determined comparing them with GeneRuler 100 bp DNA ladder (Thermo Scientific, USA) using Gel analyzer plug-in on the Image J software. Then, all the isolates with the same banding pattern were grouped. The distinct banding patterns were compared with the data base from the platform yeast-id.org in order to presumptively identify isolates at species level (Esteve-Zarzoso *et al.*, 1999).

To corroborate the identification by RFLP, representative isolates of each group (one every five with common pattern) were selected or sequencing. Briefly, DNA was extracted and PCR amplicon of ITS region was obtained as previously mentioned, then separated from remaining primers on a 1.5 %

agarose gel electrophoresis; the band was excised from the gel and purified using Illustra GFX PCR DNA and Gel Band purification kit (GE Healthcare, Mexico). Purified amplicons on TE buffer were sent to Macrogen Korea (ABI 3730XL 96-capillary DNA analyzer, Applied Biosystems), and then sequence was compared against nucleotide collection of NCBI BLASTN excluding uncultured and model microorganisms sequences, limiting the search on Type material. The sequences were also compared against Mycobank.org databases. In the cases that the RFLP identification of any strain was different from that obtained by sequencing, it was sent for verification by sequencing.

In vitro selection assays

The isolates previously reported as potentially useful on co-inoculation (Ortiz-Barrera *et al.*, 2015) were selected for the following phenotypic evaluation.

β-glucosidase activity

The isolates were streaked by triplicate on esculin glycerol agar plates and incubated at 25 °C for 72 h. Those with β-glucosidase activity hydrolyzed the substrate and a dark brown precipitate was observed around the colony (Pérez *et al.*, 2011).

Killer activity

The killer sensitive strain AH22 (ATCC38626) was pour plated in modified NYDA (methylene blue 0.02%, NaCl 1 % and phosphate-citrate buffer 0.5 M, pH 4.6-4.8) at a final concentration of 5 Log CFU/mL. A volume of 50 μL of overnight culture of isolates to be evaluated were drop inoculated by triplicate and incubated at 25 ºC for 72 h (Lopes and Sangorrin, 2010). Isolates were considered killer when an inhibition halo around the colony limited by a blue border was observed.

Sugar, sulfur dioxide and ethanol tolerance

The growth' kinetics of yeasts were measured using a Varioskan (Thermo Fisher Scientific, USA) turbidimetric analyzer. A solution of NYDB (broth with the same composition of NYDA, without agar), at pH 3.5, was prepared and adjusted with: a) sucrose at 25 °Brix, b) 6 % ethanol, or c) 30 mg/L of sulfur dioxide (SO_2) using a solution of potassium metabisulfite. A total of 200 μL of each medium was added to 96-well plates, and the different yeast isolates were separately inoculated by triplicate at a concentration of $1x10^5$ CFU/mL and incubated at 25 °C. Low agitation was carried out previously to measure the absorbance at 600 nm every 2 h and the slope of the exponential phase was estimated (Miranda *et al.*, 2017).

Winemaking assays

Crushed grapes cv. 'Tempranillo' were pasteurized at 60 °C during 20 min (Ribéreau-Gayon *et al*., 2006) and 700 mL were distributed in 1 L flasks. The must presented the following characteristics: 24.2 °Brix, a total titratable acidity of 7.2 g/L of tartaric acid, and pH of 3.82. Musts were first inoculated separately with the n-S isolates corresponding to species reported in fermented beverages, or used in mixed fermentations at a concentration of 1×10^5 CFU/mL and, after 24 h, with *S. cerevisiae* N05 at 1×10^6 CFU/mL. Fermentation was performed at 25°C, until must density decreased to 1000. Yeast cell viability was measured every 24 h during fermentation, for *Saccharomyces* in NYDA, and for n-S in lysine medium (Ortiz-Barrera *et al*., 2015). At the end of fermentation, pH, alcohol strength, total titritable acidity, volatile acidity, residual sugars and total SO_2 (OIV, 2019b) were measured. Three replicates for each treatment were evaluated.

Statistical analysis

A unifactorial completely randomized design was used for yeast isolates on their tolerance to sugar, ethanol and SO_2, as well as for the winemaking assays. In order to establish differences among isolates and evaluate their winemaking performance, ANOVA and Tukey test (p≤0.05) were performed with the obtained data, using the Statistical Program JMP version 11.

RESULTS AND DISCUSSION

Quantification of yeast in the different grape organs

Quantification of yeast in fruit set were less than 1 Log CFU/g, while fruits in veraison reached 3 Log CFU/g and ripe fruits 3.7 Log CFU/g averages (Table II). Concerning yeast populations in leaves according to their position along the stem, higher viable concentration on basal leaves with respect to medium and apical leaves were obtained in the distinct sampling dates and an increase of yeasts in the leaves through the time was also observed, reaching values higher than 5 Log CFU/g in October.

The values obtained in this study for immature fruits are comparable to those reported by Fleet (2003), ranging from 1 to 3 Log CFU/g, and for healthy ripe fruits, according to Barata *et al*. (2012) and Mendes *et al*. (2017). The differences on yeast viable concentration in the leaves according to their location in the stem could be due to that basal leaves are the oldest, having a longer time of exposure to the vineyard's environment than the others (Pinto *et al*., 2014). The increase of yeast viable concentration over the time on medium and basal leaves coincides with the use of chemical fungicides in this vineyard, since the application of these products is paused two weeks before ripening, allowing a major accumulation of yeasts. However, many other factors, as topographic and climatic conditions, cultivar and viticultural practices performed in the vineyard affect yeast populations (Cordero-Bueso *et al*., 2011; Bokulich *et al*., 2013).

Table II

Yeast viable concentration (Log CFU/g) on grapevines' organs cv. 'Tempranillo' established in Los Cues, Mexico

Concentração de leveduras viáveis (Log UFC/g) em órgãos de videiras da casta 'Tempranillo' instaladas em Los Cues, México

Sampling month	Inflorescence	Fruit set	Veraison fruit	Ripe fruit	Apical leaf	Medium leaf	Basal leaf	Latent Bud
May	2.1 ± 0.44	-	-	-	-	-	-	-
June	-	0.8 ± 0.54	-	-	2.7 ± 0.21	2.5 ± 0.19	3.1 ± 0.13	-
July	-	-	3.5 ± 0.18	-	-	3.5 ± 0.27	3.7 ± 0.16	-
August	-	-	-	3.7 ± 0.11	-	3.0 ± 0.04	4.0 ± 0.09	-
September	-	-	-	-	-	3.8 ± 0.19	3.8 ± 0.10	-
October	-	-	-	-	-	5.3 ± 0.13	5.5 ± 0.08	5.0 ± 0.20

Triplicate data is presented as mean ± standard deviation.

Identification of yeast species

From 93 isolates obtained and their grouping according to their restriction similarity, 14 different banding patterns were found, which corresponded to 10 species identified by PCR-RFLP, while four banding patterns did not match those on the yeast-id.org database (Table III).

Table III

Banding patterns and identification of yeast by PCR-RFLP

Padrões de bandas e identificação de leveduras por PCR-RFLP

Number of isolates	PCR product (pb)	*HhaI* (pb)	*HaeIII* (Pb)	*HinfI* (Pb)	Yeast species *Current name* (*synonym or former name*)
33	600	100 + 180 + 200	150 + 450	120 + 170 + 280	*Aureobasidium pullulans*
1	410	180 + 220	410	190 + 210	*Diutina catenulata*
15	650	300 + 350	60 + 90 + 500	130 + 245 + 275	*Filobasidium magnum* (*Cryptococcus magnus*)
14	490	230 + 260	490	240 + 250	*Hannaella luteola* (*Cryptococcus luteolus*)
7	630	300 + 330	60 + 70 + 500	120 + 160 + 350	*Naganishia albida* (*Cryptococcus albidus*)
2	630	300+330	60 + 70 + 500	280 + 350	*Cryptococcus* sp
1	550	260 + 290	100 + 375	270 + 270	*Papilotrema laurentii*
1	445	70 + 90 + 150	80 + 330	190 + 250	*Pichia fermentans*
3	550	100 + 200 + 250	50 + 500	50 + 250 + 250	*Cyberlidnera petersonii*
2	625	90 + 220 + 300	215 + 405	70 + 215 + 340	*Rhodotorula mucilaginosa*
10	550	200 + 260	550	180 + 300	Unidentified
1	600	600	80 + 520	140 + 220 + 290	Unidentified
1	630	300 + 350	500	180 + 320	Unidentified
2	600	290 + 320	110 + 400	60 + 110 + 160 + 280	Unidentified

The sequencing of a representative isolate of each one of the 14 restriction banding patterns and their comparison with the nucleotide collection of NCBI led to the identification of 11 isolates at species level and three at genus level (*Cryptococcus*), all coinciding to species previously reported as abundant on grape surfaces (Zanol *et al.*, 2010) - Table IV. From the 10 species identified by PCR-RFLP, seven coincided in the identification by both methods and three resulted on different species (*Diutinacatenulata* vs. *Clavispora* cf. *opuntiae*, *Pichia fermentans* vs. *Pichia* cf. *kluyveri* and *Cyberlidnera petersonii* vs. *Papiliotrema rajasthanensis*. The three isolates identified as *Cyberlidnera petersonii* by PCR-RFLP and later identified as *Papiliotrema rajasthanensis* by sequencing of one isolate, were confirmed by sequencing the other two isolates, all resulting in the same species. In the case of the other two different identifications, PCR-RFLP group was constituted by only one isolated each (Table III).

Then there was a subtle discrepancy between the two methods used, which is ascribed to the fact that RFLP is a more general methodology (polymorphism patterns) identifying the yeasts only presumptively. Nucleotide sequencing should be considered as a

more reliable methodology, since it is more precise at the moment of searching the homology of the sequence in the databases (Li *et al*., 2010).

Table IV

Comparison of the identity of isolates with different restriction banding patterns obtained by PCR-RFLP and sequencing

Comparação da identidade dos isolados com diferentes padrões de bandas de restrição obtidos por PCR-RFLP e sequenciação

Identification of isolate by PCR-RFLP *Current name* *(Synonym or former name)*	Identification of the isolate by sequencing *Current name* *(Synonym or former name)*	Query coverage	e-value	Identity
Aureobasidium pullulans	*Aureobasidium* cf. *melanogenum* (*A. pullulans* var. melanogenum)	98%	0	97%
Diutina catenulata	*Clavispora* cf. *opuntiae*	91%	2×10^{-148}	96%
Filobasidium magnum (*Cryptococcus magnus*)	*Filobasidium magnum* (*Cryptococcus magnus*)	98%	0	100%
Hannaella luteola (*Cryptococcus luteolus*)	*Hannaellaluteola* (*Cryptococcusluteolus*)	99%	0	100%
Naganishia albida (*Cryptococcus albidus*)	*Naganishia albida* (*Cryptococcus albidus*)	100%	0	100%
Cryptococcus sp	*Cryptococcus* sp	100%	0	99%
Papiliotrema laurentii	*Vishniacozyma carnescens* (*Cryptococcus carnescens*)	100%	0	100%
Pichia fermentans	*Pichia* cf. *kluyveri*	99%	3×10^{-179}	97%
Cyberlindnera petersonii	*Papiliotrema rajasthanensis* *Cryptococcus rajasthanensis*)	100%	0	100%
Rhodotorula mucilaginosa	*Rhodotorula mucilaginosa*	99%	0	100%
Not identified	*Papiliotrema flavescens* (*Cryptococcus flavescens*)	99%	0	99%
Not identified	*Cryptococcus* sp	99%	0	99%
Not identified	*Cryptococcus* sp	100%	0	100%
Not identified	*Rhodotorula nothofagi*	100%	0	100%

e-value: Number of expected hits that could be found just by chance on a particular size database.

On the other hand, the absence of the *Saccharomyces* genus can be attributed to the sampling carried out in a young vineyard, located far from other vines and in absence of a winery, where *S. cerevisiae* prevails (Pérez-Martín *et al*., 2014; Mendes *et al*., 2017). Another reason that may explain why no *Saccharomyces* isolates were detected is that only healthy grapes were sampled. Barata *et al*. (2012) recovered *S. cerevisiae* yeasts in post-mature decayed or damaged grapes.

The percentages of relative abundance of the different yeast species found in each organ are shown in Figure 1. *Aureobasidium* cf. *melanogenum* predominated throughout the sampling, especially in inflorescences and in the different stages of fruit development. *Pichia* cf. *kluyveri* was found in basal leaves and *Clavispora* cf. *opuntiae* in medium leaves. Finally, *Rhodotorula mucilaginosa* and *R. nothofagi* were only found in leaves.

These results coincide with those of Grube *et al*. (2011) who isolated *Aureobasidium pullulans* from stems, leaves and grape berries in 26 %, 41 % and 18 % respectively, while Garijo *et al.* (2011) found it predominately in the air of the vineyard during two consecutive years. In this study, *Naganishia albida* and *Papiliotrema flavescens,* former *Cryptococcus albidus and C. flavescens*, respectively, were located in berries, coinciding with Li *et al*. (2010) who

detected the last one in the same organ. The species prevalent in the vineyard are highly oxidative yeasts which do not survive in the anaerobic conditions of must (Varela and Borneman, 2016), however they have been reported as biocontrol agents against diverse fungal diseases (Varela and Borneman, 2016; Wang *et al*., 2018a).
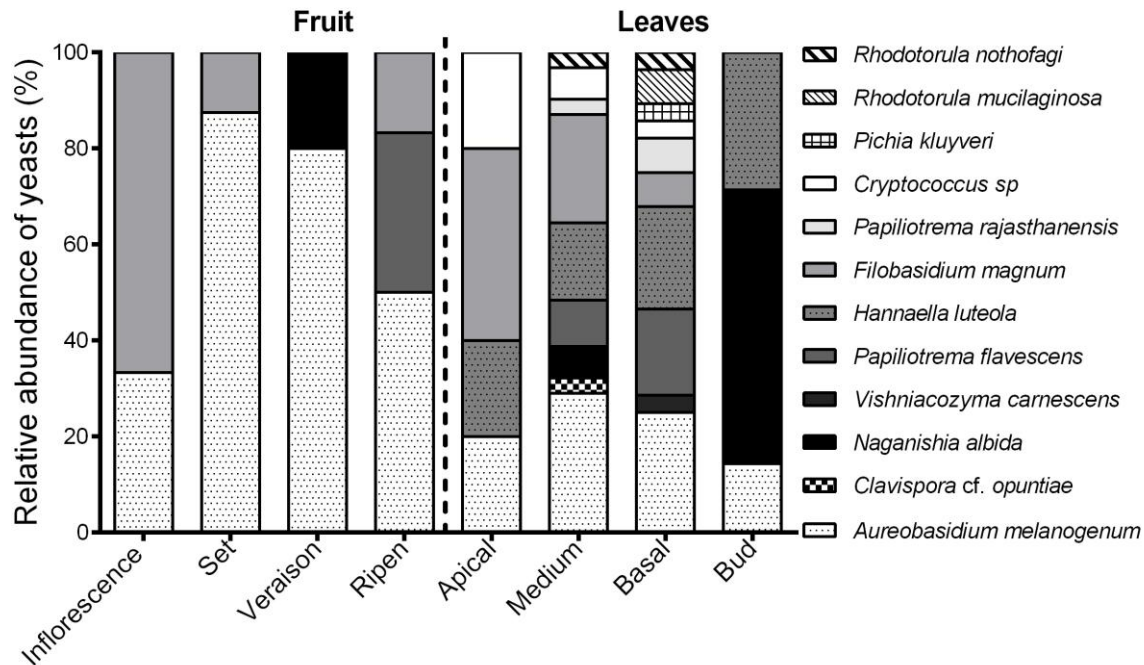

Figure 1. Relative abundance and distribution of the native yeast isolates found in the different grapevine organs.

Abundância relativa e distribuição de isolados de leveduras nativas encontrados nos diferentes orgãos da videira.

Another species found was *Pichia* cf. *kluyveri,* whose use in winemaking has been previously reported by Ma *et al*. (2017), who evaluated the synthesis of volatile compounds during the co-inoculation of a *P.* cf. *kluyveri* with *S. cerevisiae*, obtaining an increased production of ethyl esters, fatty acids, among others. Concerning *R. mucilaginosa*, Hu *et al*. (2016) reported that this species possesses β-glucosidase activity that could promote the release of glycosylated terpens associated with floral aroma. On the other hand, regarding *Clavispora* genus, *C. lusitaniae* has been isolated from Mexican fermented beverages as 'mezcal' and 'sotol' (De La Garza-Toledo *et al*., 2008; Escalante-Minakata *et al*., 2008). In the present study, the identification of this isolate should be taken with caution, considering the low query coverage (91%) and identity (96%).

For the assays regarding winemaking potential, only those isolates belonging to such species previously reported to modify wine quality in mixed fermentation were included, namely *P.* cf. *kluyveri*, *C.* cf. *opuntiae*, *R. mucilaginosa* and *R. nothofagi* (Phaff *et al*., 1986; Wang *et al*. 2017; Wang *et al*. 2018b).

In vitro selection tests

Killer activity and β-glucosidase activity

Isolates 8HM, 4.7HM and 2HE belonging to *Rhodotorula* genus showed both killer activity and β-glucosidase activity (Table V), while *P.* cf. *kluyveri* 3.1HM showed killer activity, but not β-glucosidase activity. Finally, both *H. uvarum* controls (NB27 and NB108) showed β-glucosidase activity, but not killer activity, which coincides with previous studies (Ortiz-Barrera *et al.*, 2015). The β-glucosidase activity could promote the release of glycolsylate terpenes associated with floral aroma, as Hu *et al*. (2016) found for other strains of *R. mucilaginosa*. Meanwhile Ma *et al*. (2017) obtained an increased production of ethyl esters, fatty acids, among others, while evaluating the synthesis of volatile compounds during the co-inoculation of a *P. fermentans* strain with *S. cerevisiae*. As mentioned in this and other previous studies, the evaluated features are strain dependent.

Sugar, total sulfur dioxide and ethanol tolerance

The tolerance of isolates to limiting factors relevant for winemaking was, as expected, strain dependant (Figure 2). Yeasts belonging to *Rhodotorula* genus were the least tolerant to the three stress conditions tested. The inability of the *Rhodotorula* genus to tolerate stressful conditions is explained by the fact that must is composed by aerobic yeasts, which could be present and disappear in the first stages of fermentation due to the combined effect of SO_2, low pH, high ethanol and limited nitrogen concentrations (Jolly *et al*., 2014). Nevertheless, it is interesting to emphasize their practically null sugar tolerance, despite the fact that, according to these authors, it is a genus normally present in must. On the other hand, *C.* cf. *opuntiae* 5.7 HE and both *H. uvarum* controls (NB27 and NB108) outstood in sugar tolerance, which agrees with their previous characterization carried by Ortiz-Barrera *et al*. (2015). Moreover, *Pichia* cf. *kluyveri* 3.1HM was the most tolerant yeast to 30 mg/L SO_2. Several authors have observed that *Pichia* sp. possesses a wide range of SO_2 tolerance (from 50 mg/L to 140 mg/L) (Barata *et al*., 2008; Wang *et al*., 2018b). Finally, *C.* cf. *opuntiae* 5.7HE was the most tolerant to 5 % ethanol, which is consistent with the fact that *Clavispora* genus has been found in two 'mezcal' distilleries (Kyrchmayr *et*

al., 2017), and has been described as a vigorous fermenter (Phaff *et al*., 1986).

Table V

β-glucosidase activity and killer activity of different yeast isolates

Atividade β-glucosidase e atividade killer dos diferentes isolados de leveduras

Species	Isolate	Activity	
		Killer	β-glucosidase
Pichia cf. *kluyveri*	3.1HM	+	-
Clavispora cf. *opuntiae*	5.7HE	-	+
Rhodotorula mucilaginosa	8HM	+	+
Rhodotorula mucilaginosa	4.7HM	+	+
Rhodotorula nothofagi	2HE	+	+
Hanseniaspora uvarum	Control NB108	-	+
Hanseniaspora uvarum	Control NB27	-	+

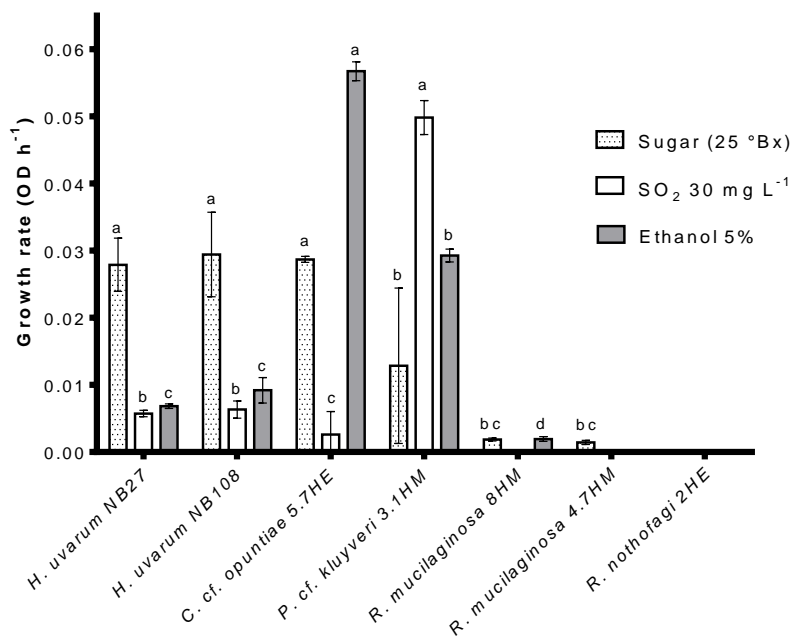(+) presents the trait; (-) does not present the trait.

Figure 2. Growth rate under different stress conditions. Triplicate data are presented as mean ± standard deviation. Bars (stress conditions) marked with the same letter are not significantly different (Tukey test, p>0.05).

Taxa de crescimento em diferentes condições de stress. Dados em triplicado, apresentados como média ± desvio padrão. Colunas (condições de stress) marcadas com a mesma letra não são significativamente diferentes (teste de Tukey, p>0,05).

Winemaking assays

The isolate showing outstanding tolerance to limit factors, *Pichia* cf. *kluyveri* (3.1HM) and *C.* cf. *opuntiae* (5.7HE), were selected for the winemaking assays. In addition, the best *R. mucilaginosa* isolate (8HM) was considered, in the hypothesis that its killer and β-glucosidase activity might produce an important effect on wine. Finally, one n-S yeast previously studied and reported (Ortiz-Barrera *et al.*, 2015), namely *H. uvarum* NB108, was used as control.

For the differential quantification of *Saccharomyces cerevisiae* N05 and n-S yeast during fermentations, lysine medium, selective for n-S, was used.
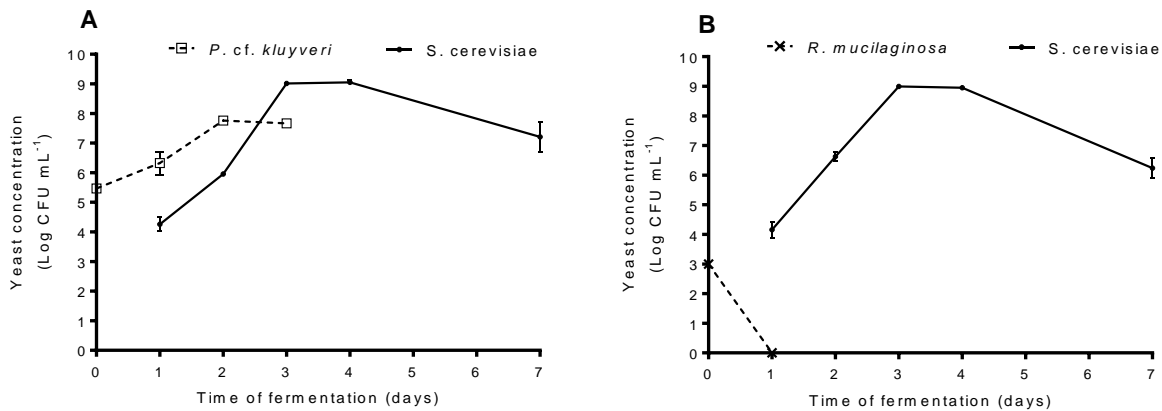
Nevertheless, *Saccharomyces* was also able to grow due to their capacity to stock nitrogen in its vacuoles (Watson, 1976). Therefore, differential quantification of yeast in this medium was only possible for *R. mucilaginosa* (salmon color) and *P.* cf. *kluyveri* (rugged), both showing contrasting morphologies with *S. cerevisiae*.

Figure 3 shows the growth kinetics for *P.* cf. *kluyveri* 3.1HM and *S. cerevisiae* N05 (PS), and that for *R. mucilaginosa* 8HM and *S. cerevisiae* N05 (RS).The differences between the inoculum and viable counts of yeast in the inoculated must could be explained by the stress caused on microorganisms by the particularly complex environment, unusual for them.

Figure 3. Growth kinetics, during fermentation, *of S. cerevisiae* N05 with *P.* cf. *kluyveri* 3.1HM (A), and with *R. mucilaginosa* 8HM (B).

Cinética de crescimento, durante a fermentação, de *S. cerevisiae* N05 com *P.*cf. *kluyveri* 3.1HM (A), e com *R. mucilaginosa* 8HM (B).

P. cf. *kluyveri* 3.1HM population increased to a final count of approximately 8 Log CFU/mL two days after its inoculation. At the same time (24 hours after its inoculation) *S.cerevisae* N05 population arrived to 5 Log CFU/mL. Afterwards, *S. cerevisiae* adapted and actively multiplied, increasing 4 Log CFU/mL in 24 h, and reaching a population close to 9 Log CFU/mL, while *P.* cf. *kluyveri* 3.1HM reached the stationary phase (Figure 3A). On the other treatment, *R. mucilaginosa* 8HM was not detected after 24 h from its inoculation and *S. cerevisiae* N05 arrived to more than 6 Log CFU/mL 24 h after its inoculation (Figure 3B). On mixed fermentations, *P. cf. kluyveri* 3.1HM rapidly adapted to the medium which coincides with its relative tolerance to SO_2, ethanol and sugar (Figure 2). Meanwhile, *R. mucilaginosa* 8HM evidenced its incapacity to adapt under must conditions (sugar,

SO_2, pH and nitrogen deficiency), previously observed in the tolerance assays (Figure 2).

Finally, the behavior of *S. cerevisiae* N05 was similar in both mixed fermentations, with a slightly lower viability 24 hours after having been inoculated in presence of *P.* cf. *kluyveri* 3.1HM, in relation to that obtained at the same time in presence of *R. mucilaginosa* 8HM, probably due to some competition offered by this n-S isolate in the first case, associated to the higher concentration of *P.* cf. *kluyveri* (~2 Log CFU/mL) than that of *S. cerevisiae* at this time. As any other organism, yeast competes for space and resources in a shared environment with other yeasts, which is observed in the mixed fermentation with *R. mucilaginosa* 8HM, where only *S. cerevisiae* prevailed, taking advantage of space and/or nutrients available, which contrast with its behavior in the presence *P.* cf. *kluyveri* 3.1HM.

Physical and chemical analyses of wines

Differences among treatments were only found for pH and volatile acidity, being the control *S. cerevisiae* N05 with *H. uvarum* NB108 (HS) the highest in both (3.75 and 0.45 g/L acetic acid, respectively) (Table VI). Differences in pH could be explained by the different concentrations of succinic, lactic, piruvic and malic acids and other acids produced by the distinct n-S yeasts during fermentation (Whiting, 1976; Chidi *et al.,* 2018). Nevertheless, no differences between treatments in TTA were apparent. The higher volatile acidity obtained in the co-inoculation of *Hanseniaspora uvarum* NB108 agrees with Jolly *et al*. (2014) who reported that *Hanseniaspora* species produce high quantities of acetic acid and ethyl acetate, which make them less attractive for winemaking. Ortiz-Barrera *et al*. (2015) performed mix fermentations with *S. cerevisiae* and different *Hanseniaspora* strains, finding values of volatile acidity lower than 0.2 g/L acetic acid, which differ from our results, probably due to differences in winemaking conditions.

Concerning the other analyzed variables, residual sugars, less than 2 g/L, and alcohol strength, higher than 13 %, correspond to dry wines and confirm complete fermentations. Moreover, total SO_2 values are under the limits accepted for quality wines (OIV, 2019a). These results show that n-S yeast evaluated in this study do not have any negative impact in the chemical characteristics of wines and could be used in commercial mix fermentations. Nevertheless, the most recognized contribution of n-S lays down on subtle sensory attributes, which seem to provide complexity and tipicity to wine (Jolly *et al.*, 2014).

Table VI

pH, total titritable acidity (TTA), volatile acidity (VA), alcohol strength, residual sugars and total sulfur dioxide of 'Tempranillo' wines elaborated with different mixed fermentations

pH, acidez total (TTA), acidez volátil (VA), título alcoométrico, açúcares residuais e dióxido de enxofre total de vinhos da casta 'Tempranillo' elaborados com base em diferentes fermentações mistas

Treatment	pH	TTA (g tartaric acid/L)	VA (g acetic acid/L)	Alcohol strength (% v/v)	Residual sugars (g/L)	Total SO_2 (mg/L)
RS	3.67 ± 0.02[bc]	7.39 ±0.11 [a]	0.22 ± 0.03[b]	12.97 ± 1.6[a]	1.85 ± 0.02[a]	117.33 ± 15.79[a]
PS	3.66 ± 0.01[c]	7.59 ± 0.11[a]	0.27 ± 0.02[b]	13.63 ± 0.47[a]	1.69 ± 0.17[a]	124.80 ± 6.40[a]
CS	3.65 ± 0.04[c]	7.62 ± 0.15 [a]	0.25 ± 0.02[b]	13.75 ± 0.62[a]	1.66 ± 0.13[a]	117.33 ± 7.39[a]
HS	3.75 ± 0.01[a]	7.59 ± 0.07[a]	0.45 ± 0.03[a]	13.98 ± 0.81[a]	1.71 ± 0.10[a]	118.40 ± 6.40[a]
N05	3.72 ± 0.02[ab]	7.44 ± 0.20[a]	0.19 ± 0.05[b]	13.16 ± 0.18[a]	1.64 ± 0.11[a]	130.13 ± 3.70[a]

Mean and standard deviation from three replicates are shown. Means followed by the same letter in a column are not significantly different (Tukey test, p>0.05). Mix fermentation treatments of *S. cerevisiae* N05 with: *R. mucilaginosa* 8 HM (RS), *P.* cf. *kluyveri* 3.1 HM (PS), *C.* cf. *opuntiae* 5.7 HM (CS); as mix control with *H. uvarum* NB108 (HS) and general control: *S. cerevisiae* alone (N05).

CONCLUSIONS

Yeast populations in grapevines vary highly depending of the organ and the sampling season, and all the isolates correspond to non-*Saccharomyces* yeasts. The prevalent genera in the vineyard are the highly oxidative genus *Aureobasidium* and basidiomycota yeasts.

Pichia cf. *kluyveri* 3.1HM showed killer activity, was the most tolerant to sulfite and survives along the exponential phase of *S. cerevisie*. *Clavispora* cf. *opuntiae* 5.7HM showed the highest tolerance to ethanol and sugar, as well as β-glucosidase activity.

Both strains should be further studied in mixed fermentations for the production of regional wines. On the other hand, the different *Rhodotorula* spp.isolates evaluated lacked tolerance to the stress conditions evaluated and R. *mucilaginosa* 8HM was not able to grow in the must environment.

This study provided insights on the diversity of yeast present in distinct parts of grapevines established in Queretaro, Mexico, and revealed the oenological potential of *Pichia* cf. *kluyveri* 3.1HM.

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