Archives of Microbiology (2023) 205:302
https://doi.org/10.1007/s00203-023-03646-1

ORIGINAL PAPER

Isolation and identification of native yeasts from the spontaneous fermentation of grape musts

Liliana Mabel Gerard[1] · María Belén Corrado[1] · Cristina Verónica Davies[1] · Carina Alejandra Soldá[1] · María Gabriela Dalzotto[1,2] · Sofía Esteche[1]

Received: 17 April 2023 / Accepted: 1 August 2023 / Published online: 7 August 2023

Abstract

Recently, there has been growing interest in the characterization of native yeasts for their use in production of wines with regional characteristics. This study aimed to investigate *Saccharomyces* and non-*Saccharomyces* yeasts present in the spontaneous fermentation of Tannat and Marselan grape musts collected from Concordia (Entre Ríos, Argentina) over 2019, 2020, and 2021 vintages. The evolution of these fermentative processes was carried out by measuring total soluble solids, total acidity, volatile acidity, pH, ethanol concentration, and total carbon content. Isolated *Saccharomyces* and non-*Saccharomyces* yeasts were identified based on colony morphology in WL medium, 5.8S-ITS-RFLP analysis, and 26S rDNA D1/D2 gene sequencing. Two hundred and ten yeast colonies were isolated and identified as *Pichia kudriavzevii, Saccharomyces cerevisiae, Hanseniaspora uvarum, Metschnikowia pulcherrima, Candida albicans, Candida parapsilosis, Pichia occidentalis, Pichia bruneiensis, Hanseniaspora opuntiae, Issatchenkia terricola,* and *Hanseniaspora vineae. P. kudriavzevii* isolated from all vintages was associated with the spontaneous fermentation of grape musts from the Concordia region.

Keywords Spontaneous fermentation · Non-*Saccharomyces* yeasts · *Saccharomyces* yeasts · Tannat · Marselan · Grapes

Introduction

Alcoholic fermentation is a complex process with many biochemical changes due to the activities of fermenting microorganisms, such as several yeast species, as well as external physical factors. Although ethanol and carbon dioxide are the main fermentation products, other compounds that influence beverage flavor and color are also produced. These products vary according to the sugar content of the raw material and yeast activity, though beverage composition may differ depending on their origin (Del Fresno et al. 2017).

During the spontaneous fermentation of grape musts, non-*Saccharomyces* yeasts develop first. They are naturally present in grapes, in greater numbers than *Saccharomyces cerevisiae,* and are adapted to the environment (Cray et al. 2013). *Hanseniaspora, Pichia, Debaryomyces, Issatchenkia, Candida,* and *Metschnikowia* stand out among the non-*Saccharomyces* genera (Jolly et al. 2014; Grangeteau et al. 2016; Padilla et al. 2016). Since *S. cerevisiae* is more tolerant to ethanol and is competitive to grow under such environmental conditions, it shows better adaptation and subsequent develops (Jolly et al. 2014; Vaudano et al. 2019). However, some non-*Saccharomyces* yeasts can survive until the end of fermentation because of their high ethanol resistance (Combina et al. 2005).

Until a few years ago, non-*Saccharomyces* yeasts were considered responsible for microbiological problems and wine defects because they were isolated from altered wines (Padilla et al. 2016). However, current researches recognize their fundamental role in winemaking processes because they provide distinctive characteristics in wines (Maturano et al. 2016; Barkhuizen et al. 2021; Cioch-Skoneczny et al. 2021; Drumonde-Neves et al. 2021). Although they are known to be poor fermenters because of their low tolerance

Communicated by Yusuf Akhter.

✉ Liliana Mabel Gerard
liliana.gerard@uner.edu.ar

[1] Laboratorio de Microbiología y Biotecnología de Alimentos, Facultad de Ciencias de la Alimentación, Universidad Nacional de Entre Ríos, Monseñor Tavella 1450, 3200 Concordia, Entre Ríos, Argentina

[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Concordia, Argentina

to ethanol, many are being investigated for winemaking purposes (Varela and Borneman 2017; Martin et al. 2018; Drumonde-Neves et al. 2021). Some non-*Saccharomyces* yeasts can be used to reduce ethanol levels in wines (Mestre Furlani et al. 2017; Maturano et al. 2019), whereas others can degrade malic acid during malolactic fermentation (del Mónaco et al. 2014). Moreover, the use of non-*Saccharomyces* yeasts in single or mixed/sequential fermentations is a powerful tool for improving the fruity aromatic quality and complexity of wines, and thus, to achieve a better definition of the regional flavor style (Padilla et al. 2016; Del Fresno et al. 2017; Shi et al. 2019; Lai et al. 2022). There is a modern approach, supported by rigorous scientific research, to apply 'multispecies' wine ferments, specifically native *S. cerevisiae* and non-*Saccharomyces* species (Jolly et al. 2014; Martin et al. 2018; Shi et al. 2019). Consequently, the use of autochthonous yeast species requires isolation and characterization procedures as well as molecular techniques for their identification.

The study of the effects of non-*Saccharomyces* yeasts on vinification is a trending topic among researchers in different countries (Del Fresno et al. 2017; Cimini and Moresi 2022). In recent years, several studies have focused on the characterization of native yeasts involved in spontaneous fermentation, mainly to understand the ecology, physiology, biochemistry, and molecular biology of *Saccharomyces* and non-*Saccharomyces* species (Maturano et al. 2016; Raymond Eder et al. 2017; Mendoza et al. 2019; Raymond Eder and Rosa 2019; Shi et al. 2019; García-Béjar et al. 2021; Zhang et al. 2021).

In Argentina, several researchers have studied the yeasts isolated from different grape varieties during spontaneous fermentation. Malbec varieties were analyzed in Cuyo (western Argentina) (Combina et al. 2005) and Patagonia regions (southern Argentina) (del Mónaco et al. 2014, 2016). In Córdoba (central Argentina), Isabella and Malbec varieties have been investigated (Raymond Eder et al. 2017; Raymond Eder and Rosa 2019), while studies in Malbec, Merlot, Syrah, and Torrontes from northern Argentina (Mendoza et al. 2019) have also been reported. However, no studies have been found on native yeasts from the east of the country (Entre Ríos, Argentina).

The province of Entre Ríos, located in eastern Argentina, is positioned as a new producer of vines and wines. Tannat and Marselan varieties are among the *Vitis vinifera* mainly cultivated in this region. The microbial communities of these grapes, particularly yeasts, have not yet been studied. Since no investigations have been reported, a special interest in their study has increased.

Many factors affect the diversity of microorganisms in grapes, such as climate, location, and grape physicochemical parameters (Combina et al. 2005; Vaudano et al. 2019; Sumby et al. 2021). This observation reinforces the interest in searching for wine yeast diversity in ecological niches alternative to traditional environments.

The aim of this study was to analyze the population dynamics of native yeasts (*Saccharomyces* and non-*Saccharomyces*) during spontaneous fermentation of Tannat and Marselan grape musts and their identification using both culture-based and molecular identification approaches.

Materials and methods

Grape sampling

Tannat and Marselan grape varieties were collected during 2019, 2020, and 2021 vintages, from a vineyard located in La Criolla (Concordia department, Entre Ríos province, latitude − 31°14′39″, longitude − 58°07′17″). Samples consisted of healthy grape bunches, not damaged, and randomly harvested at their optimal ripeness, across three vineyard lines. Bunches were placed in sterile bags, transported to the laboratory under cold storage, and maintained at 5 ± 2 °C until assay development.

Yeast isolation and cell count. Macroscopic and microscopic characteristics

Native yeast counts from Tannat and Marselan grapes were determined according to the methodology described below. 200 g of each variety were destemmed and aseptically crushed in a stomacher (IUL Instruments, Spain) for 20 s. Musts were supplemented with 85 mg/L sodium metabisulfite (Cicarelli, Argentina) and incubated at 25 ± 2 °C for 12 days to allow spontaneous fermentation. Sample aliquots were taken regularly, and suitable dilutions were plated in duplicate in order to yeast count. YPD agar (1% yeast extract (Britania, Argentina), 2% peptone (Britania), 2% dextrose (Biopack, Argentina), 1.5% agar (Britania) with 30 µg/mL chloramphenicol (Merck, Germany) (YPDC) was employed for total yeast counts while YPDC agar with 0.4 µg/mL cycloheximide (Merck) (YPDCL) to inhibit *Saccharomyces,* was used for non-*Saccharomyces*. Plates were incubated at 25 ± 2 °C for 72 h.

Yeast isolation was carried out on Wallerstein Laboratories (WL) differential nutrient agar (Oxoid, England) supplemented with 30 µg/mL chloramphenicol. Volumes of 0.1 mL from serially diluted samples were plated in duplicate. After 72 h of incubation at 25 ± 2 °C, yeast colonies showing different phenotypes (morphology and/or color) were isolated and cultured on WL agar to obtain a pure culture. From each grape variety and vintage, 27–41 representative colonies of all morphologies were selected. The microscopic characteristics (morphology, budding, etc.) were observed using an optical microscope (Leica, USA) at 100 × magnification.

For long-term storage, yeast cells were inoculated into YPD broth, incubated for 48 h at 25 ± 2 °C, and then frozen at -80 °C using sterile glycerol (15% v/v) (Biopack) as a cryoprotective agent.

Monitoring of spontaneous alcoholic fermentation

The evolution of spontaneous fermentative processes in Tannat and Marselan grapes was carried out simultaneously with yeast counts. The fermenting musts were previously described, and the following physicochemical parameters were periodically determined over a 12-days period:

Total soluble solids

Refractometric method with a Hanna HI 96801 refractometer (Romania). Results were expressed as °Brix.

Total acidity

Potentiometric titration with sodium hydroxide (Cicarelli), according to MA-E-AS313-01: R2015 OIV technique (2020). Results were expressed as g tartaric acid/L.

Volatile acidity

Steam distillation (Jaulmes method), according to MA-E-AS313-02: R2015 OIV technique (2020). Results were expressed as g acetic acid/L.

pH

Potentiometric method with a BOECO BT-500 pHmeter (Germany), according to MA-E-AS313-15: R2011 OIV technique (2020).

Ethanol concentration

Enzymatic method (Boehringer Mannheim/R-Biopharm, Cat. N° 10,176,290,035, Germany). Results were expressed as % (v/v).

Total carbon concentration

Dumas method, dry digestion, and quantification with LECO CHN 628 according to OMA (2019). Results were expressed as g/100 g dry matter.

Molecular identification

Standard strain: *S. cerevisiae* ATCC 9763 provided by Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) "Dr. Carlos G. Malbrán" (Argentina) was used as a standard strain for molecular assays. Species identification was carried out using the Yeast-ID.org database (https://www.yeast-id.org/) based on restriction analysis of the region including the gene codifying 5,8S rRNA and the transcribed intergenic regions ITS (5,8S-ITS).

DNA extraction

Each strain was cultured in test tubes containing 10 mL of YPD broth at 30 ± 1 ºC for 48 h. A volume of 1 mL was centrifuged at 2400 g for 10 min and DNA was extracted according to the CTAB method (Wilson 2001). DNA was visualized by electrophoresis on 1% (w/v) agarose gel (Genbiotech, Argentina) in 1 × TBE buffer at 100 V for 60 min. Gels were stained with 0.5 µg/mL ethidium bromide (Genbiotech) and visualized under UV light (Labnet International, Inc. USA). A 1 kb molecular weight marker was used (Genbiotech, Argentina). DNA was stored at − 20 ± 1 °C until use.

PCR amplification and analysis

All isolated strains were identified by PCR amplification of the 5.8-ITS rDNA region using ITS1 and ITS4 primers (White et al. 1990). DNA amplifications were carried out in 40 µL final volume containing 0.3 U GoTaq G2 DNA polymerase (Promega®, USA), 1 X PCR reaction buffer, 0.4 mM dNTP, 0.6 µM from each primer, 2 µL DNA (50–100 ng/µL). The PCR was performed on a Longgene MG96G (China) thermal cycler, under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 2 min, extension at 72 °C for 2 min, and a final extension step of 10 min at 72 °C. PCR amplification products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel (Genbiotech) in 1 × TBE buffer, separated at 100 V for 100 min. Gels were stained with 0.5 µg/mL ethidium bromide (Genbiotech) and visualized under UV light. A 100 kb molecular weight marker was used (Genbiotech).

Restriction analysis

PCR products were digested with the restriction endonucleases *Cfo*I, *Hae*III, and *Hinf*I (Pham et al. 2011) according to the manufacturer's instructions (Promega®). Restriction fragments were separated on a 2% (w/v) agarose gel (Genbiotech) at a constant voltage of 80 V for 150 min and stained with ethidium bromide. A 25 pb molecular weight marker (Inbio Highway, Argentina) was used and species

assignations were performed by comparison with profiles recorded in the Yeast-ID database.

26S rDNA D1/D2 gene sequencing and sequence analyses

In order to confirm the found species, some isolates, representative of each identified profile, were selected and the D1/D2 domain was sequenced from the 26S rDNA and amplified by NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCG TGTTTCAAGACGG-3′) primers (O´Donnel et al. 1993), using the PCR conditions described by Wang and Liu (2013). PCR products (600 bp) were sent for purification and subsequent sequencing (Macrogen Inc., Seoul, Korea) and the results were compared with those available in the NCBI GenBank nucleotide sequence database (http://www.ncbi.nlm.nih.gov/genbank/). Sequences from the representative strains were then deposited in the database with accession numbers.

Yeast diversity

The percentage distribution of yeast species isolated from Tannat and Marselan grapes was calculated by comparing the number of species detected with the total isolated yeasts per vintage and grape variety. It was calculated as follows: $\% = NS/NT \times 100$, where NS is the total strain per species and NT is the total isolated yeast.

Results and discussion

Dynamic of yeast populations in spontaneous fermentation of Tannat and Marselan grape musts

The population dynamics of *S. cerevisiae* and non-*Saccharomyces* yeasts for Tannat and Marselan grapes, during the 2019, 2020, and 2021 vintages, are shown in Fig. 1. The difference between the total yeast counts in YPDC agar and YPDCL agar indicates the relative contribution of *S. cerevisiae.* Initially, the total yeast count reached a population of 10^3 CFU/mL, similar to non-*Saccharomyces* yeast. These values are comparable to those reported by other authors (Combina et al. 2005; Maturano et al. 2016; Zabukovec et al. 2020). However, unexpectedly, for both varieties in the 2020 vintage, *S. cerevisiae* was observed at the beginning of fermentation. This was in accordance with Maturano et al. (2016), who found *S. cerevisiae* in large quantities (39%) in grape must. As fermentation time advanced, total yeast counts increased up to 10^6 CFU/mL, and simultaneously, *S. cerevisiae* showed a proliferation in their population to the detriment of non-*Saccharomyces* yeasts. Although non-*Saccharomyces* yeasts decreased in number during fermentation, they remained viable until the end of fermentation.

Although only a few researchers have carried out *S. cerevisiae* and non-*Saccharomyces* yeast counts on YPDCL and YPDC agar during musts spontaneous fermentation, they agreed that only *S. cerevisiae* was identified at the final stages (Combina et al. 2005; Raymond Eder et al. 2017; Zabukovec et al. 2020). It is important to note that *Pichia kudriavzevii* (a non-*Saccharomyces* species) was also identified in this research work at the end of fermentation assays and in large counts, probably due to its ethanol resistance (experimentally demonstrated but not shown). Nieto-Sarabia et al. (2022) reported similar results.

Identification of yeast species

Yeast species from spontaneously fermenting musts of Tannat and Marselan grapes harvested in 2019, 2020, and 2021 vintages were isolated and identified. Two hundred and ten (210) colonies of native yeasts were isolated: 34, 35, and 41 from Tannat variety and 27, 38, and 35 from Marselan, respectively. Initially, yeast colonies were analyzed according to their morphology and color on WL nutrient agar in addition to microscopic observations (Table 1). Cavazza et al. (1992), Pallmann et al. (2001), Polizotto et al. (2016) and Li et al. (2018) reported that most yeast species typically found in grape musts fermentation could be differentiated according to their morphology and/or colony color on WL medium. However, it was observed that both characteristics in this medium were modified over time. As can be seen in Fig. 2 (VII a, b), *C. parapsilosis* initially formed pale green colonies with a white rim, glossy, and after 7 days, the color turned to emerald green.

According to Wang and Liu (2013), some *I. terricola* strains exhibited pale green colonies with white rims, surfaces with circular dents, and consistency of flour, whereas others were white with a hint of yellow, surface with circular dents, and consistency of flour. In the present study, colonies of these strains were green, black in the center, convex, and had an elevated dome. Nevertheless, WL agar is very useful for the preliminary differentiation of colonies prior to molecular identification.

To the best of our knowledge, macroscopic characteristics of some isolated yeasts such as *H. opuntiae*, *P. occidentalis and P. bruneiensis* grown on WL agar, have not been previously described (Table 1, Fig. 2). The first two species have been often found in grape musts and wines (Drumonde-Neves et al. 2021). Some authors have isolated *P. bruneiensis* from *Hibiscus* flowers (Sipiczki 2012) and apples (Liu et al. 2022) but nothing has been found in grapes, wines, or vineyards.

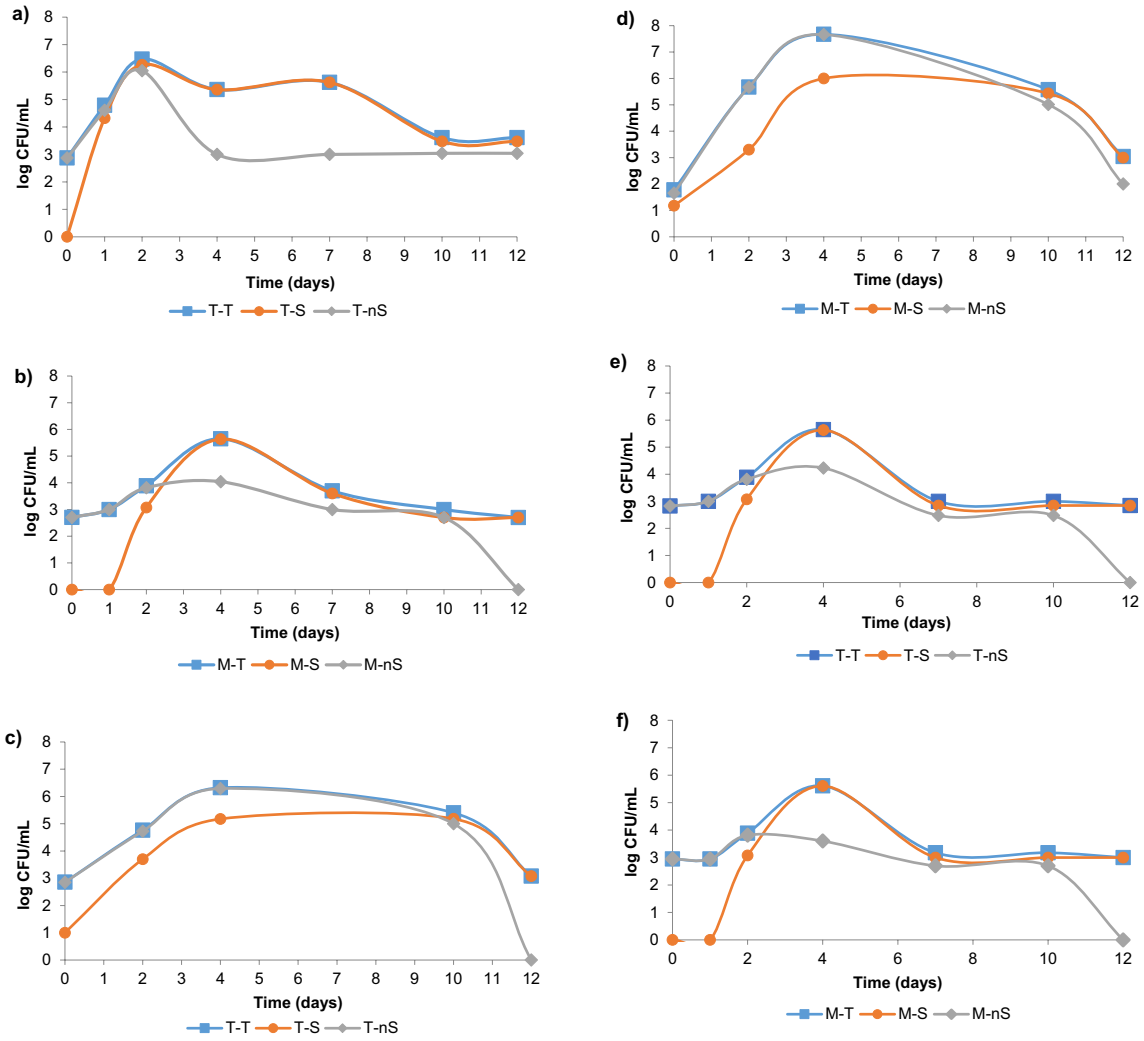

Fig. 1 Spontaneous fermentation of grape musts. Population dynamics of Tannat yeasts from the 2019 vintage (**a**), 2020 vintage (**c**), and 2021 vintage (**e**): total yeasts (T–T), *S. cerevisiae* (T–S), and non-*Saccharomyces* yeasts (T-nS). Population dynamics of Marselan yeasts from the 2019 vintage (**b**), 2020 vintage (**d**), and 2021 vintage (**f**): total yeasts (M–T), *S. cerevisiae* (M–S), and non-*Saccharomyces* yeasts (M-nS)

Since *P. kudriavzevii* exhibited white and opaque colonies, very similar to *S. cerevisiae*, its differentiation became too difficult (Fig. 2). Therefore, during the middle and final stages of yeast isolation, and according to the morphology observed on WL agar, spontaneous fermentation was thought to be dominated by *S. cerevisiae*. However, molecular identification assays also detected *P. kudriavzevii*. In addition, the growth of this species in tubes with broths showed a different behavior because it formed white agglomerated particles on the tube's wall, above the liquid surface (up to 2 cm). These characteristics have not been reported previously.

Isolated yeasts were subjected to molecular analysis using the PCR method of the internal transcribed spacer region (ITS), which comprises 5.8S rRNA and two flanking regions (ITS1 and ITS2) (White et al. 1990). The isolates showed different PCR product sizes ranging from 380 to 880 bp (Table 1). Subsequently, the products were digested with *Cfo*I, *Hae*III, and *Hinf*I restriction enzymes (Pham et al. 2011). Digestion with each endonuclease yielded eleven different restriction profiles (Table 1). Isolated species were mostly differentiated based on these patterns. However, due to a high level of homology between groups VIII and IX, their differentiation was not possible with the aforementioned restriction enzymes. The cited patterns belong to three *Hanseniaspora* species: *H. guillermondi, H uvarum, and H. opuntiae* (Garofalo et al. 2016; Wang et al. 2019). Some authors have reported the possibility of using the *Dde*I and *Mbo*II restriction enzymes (Nisiotou et al. 2007; Wang et al. 2019). However, they were not available in the

Table 1 Macroscopic and microscopic characteristics, PCR products, restriction fragments and sequencing analysis of yeasts isolated from Tannat and Marselan grapes

Isolated yeast group (N)	Morphology		PCR-ITS (bp)	Restriction fragments (bp)			Yeast species by PCR–RFLP	D1/D2 domain of 26S rDNA sequence (% Identity)/% Query coverage/accession number
	Macro	Micro		*Hinf* I	*Cfo*I	*Hae*III		
I (6)	White, flat and well defined rim and surface, consistency of cream	Round and globose	380	190–180	220–95–80	280–100	*M. pulcherrima*	*M. pulcherrima* (99.79%)/(98%)/OQ553803
II (2)	Green, black in the center, convex and with elevated dome	Oval	420	230–100–100	130–100–90–85	280–120	*I. terricola*	*I. terrícola* (98.03%)/(94%)/OQ520340
III (1)	Cream with white rim, consistency of cream	Apiculate	450	280–100-90	250–100-90	320–80	*P. occidentalis*	*P. occidentalis* (100%)/(98%)/OQ553931
IV (2)	Olive green, black in the center	Oval	450	280–220	150–100–80	310–110	*P. bruneiensis*	*P. bruneiensis* (98.14%)/(98%)/OQ559391
V (73)	White, opaque, irregular surface, convex	Round	520	220–160–140	220–180–75–50	400–100	*P. kudriavzevii*	*P. kudriavzevii* (99.31%)/(83%)/OQ520881 *P. kudriavzevii* (99.64%)/(100%) /OQ553797
VI (3)	Cream, glossy, convex with gray-green rim	Elongated	550	280–270	290–260	450–100	*C. albicans*	*C. albicans* (94.04%)/(98%)/OQ553801
VII (2)	Palegreen with a white rim and glossy After six days, the color was emerald green	Elongated	550	260–240	310–250	420–120	*C. parapsilosis*	*C. parapsilosis* (99.82%)/(82%)/OQ521663
VIII (7)	Dark green, regular rim	Elongated	760	350–200-180	310–300-100	760	Not identified	*H. opuntiae* (99.65%)/(94%)/OQ521667 *H. opuntiae* (97.21%)/(83%)/OQ520564

Table 1 (continued)

Isolated yeast group (N)	Morphology		PCR-ITS (bp)	Restriction fragments (bp)			Yeast species by PCR–RFLP	D1/D2 domain of 26S rDNA sequence (% Identity)/% Query coverage/accession number
	Macro	Micro		*Hinf* I	*Cfo*I	*Hae*III		
IX (28)	Intense green, regular rim, flat, smooth, opaque surface, cream consistency	Pointed	760	370–190–170	320–315–100	760	Not identified	*H. uvarum* (97.58%)/(83%)/OQ520337
X (2)	Pale green, regular rim	Round	770	380–350	260–150-140	650–80	*H. vineae*	*H. vineae* (100%)/(100%)/OQ550975
XI (84)	White, smooth surface and rim	Globose to ovoidal	880	370–365–140	380–365–140	320–220–180–145	*S. cerevisiae*	*S. cerevisiae* (95.76%)/(81%)/OQ520880 *S. cerevisiae (98.7%)/(100)/OQ553805* *S. cerevisiae (98.57%)/(99%)/OQ559564* *S. cerevisiae (99.48%)/(80%)/OQ521665*
XI	White, smooth surface and rim	Globose to ovoidal	880	370–365–140	380–365–140	320–220–180–145	*S. cerevisiae ATCC 9763*	Not sequenced

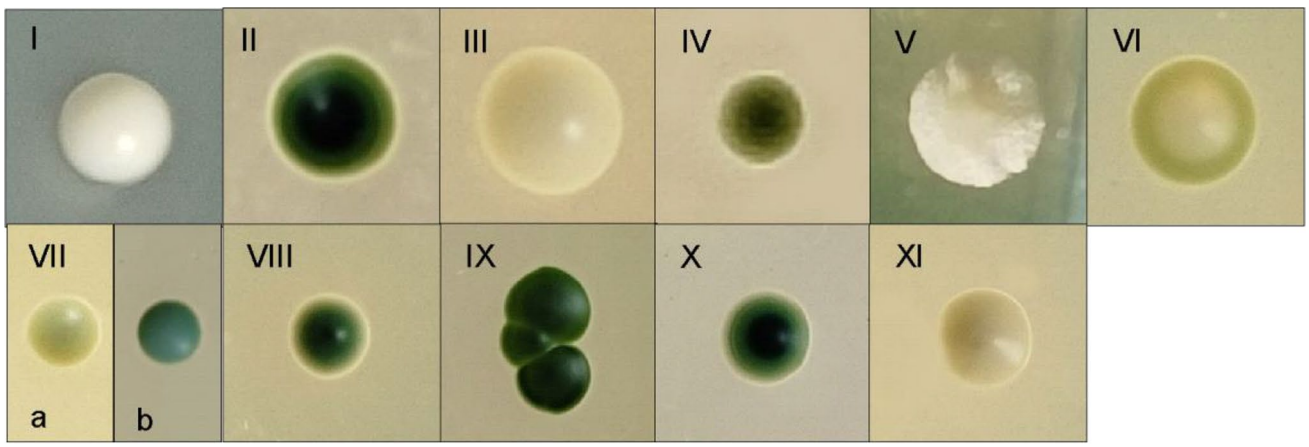

Fig. 2 Photographs of yeast colony morphotypes on WL nutrient agar. I: *M. pulcherrima*; II: *I. terricola*; III: *P. occidentalis*; IV: *P. bruneiensis*; V: *P. kudriavzevii*; VI: *C. albicans*; VII: *C. parapsilosis*, a: 72 h. after inoculation, b: 7 days after inoculation; VIII: *H. opuntiae*; IX: *H. uvarum*; X: *H. vineae*; XI: *S. cerevisiae*

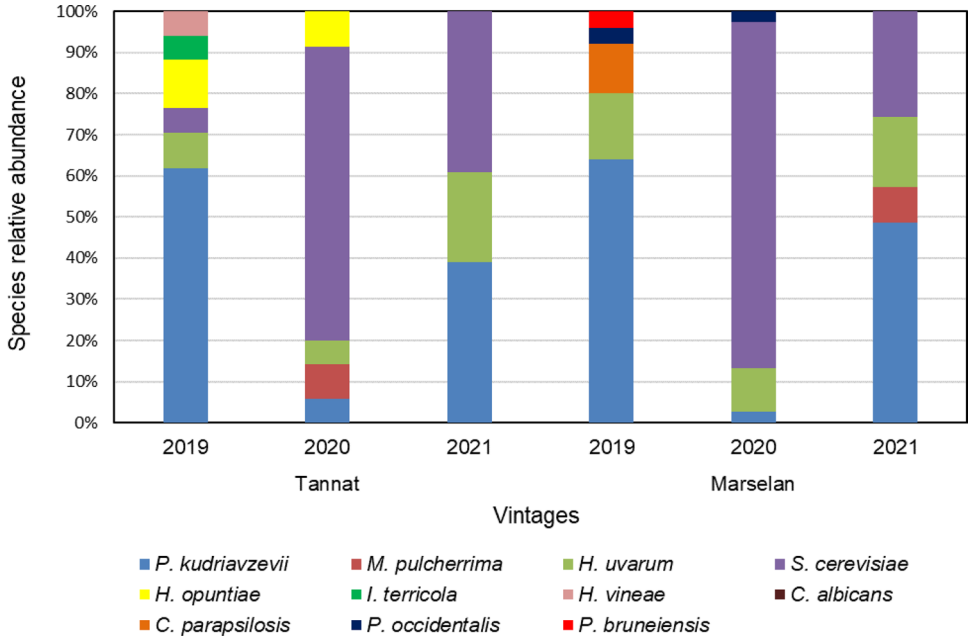

Fig. 3 Yeast community diversity during spontaneous fermentation of Tannat and Marselan grape musts. Percentages represent the relative contribution of each species to the total number of yeast isolates at different vintages

laboratory, therefore, identification by sequencing the 26S rDNA D1/D2 domain genes was necessary.

Similar diversity of non-*Saccharomyces* yeasts was observed in both varieties and also in all analysed vintages during the first stages of fermentation (Fig. 3). *S. cerevisiae* and *P. kudriavzevii* were the most dominant species. They contributed 40% and 35% of all isolates in both varieties, respectively, followed by *H. uvarum* (13%). Other non-*Saccharomyces* species were less frequently identified (Fig. 3). *M. pulcherrima* was isolated from both varieties, whereas *P. occidentalis, P. bruneiensis, C. albicans,* and *C. parapsilosis* were found only in Marselan, and *H. opuntiae*, *I. terricola,* and *H. vineae,* in Tannat grapes. Some of these yeast species (i.e., *H. uvarum, H. vineae*, *C. albicans, P. opuntanie, C. parapsilosis, I. terricola,* and *P. kudriavzevii*) have been widely described in grapes from other regions (Raymond Eder et al. 2017; Guaragnella et al. 2020; Zabukovec et al. 2020; Drumonde-Neves et al. 2021).

It is well known that *S. cerevisiae* is the dominant species in spontaneous fermentation of grape musts. However, only a few studies have recognized *P. kudriavzevii* as a fermenting species suitable for winemaking processes (Aponte and Giuseppe 2016; del Mónaco et al. 2016; Shi et al. 2019).

For all three vintages, *H. uvarum* was the third most abundant species in Tannat and Marselan varieties. Several studies have reported its presence in both grapes and musts (Maturano et al. 2016; Vaudano et al. 2019; Drumonde-Neves et al. 2021).

Sequencing analysis

All isolates identified by PCR–RFLP patterns were consistent with the sequencing results. However, some yeasts (genus *Hanseniaspora*) could not be differentiated in the ID Yeast database because they produced similar patterns to the assayed enzymes. Therefore, they can only be identified by sequencing.

Sequences obtained were uploaded to the NCBI GenBank nucleotide sequence database, and the following accession numbers were obtained: Group I, OQ553803 (99.79%); Group II, OQ520340 (98.03%); Group III, OQ553931(100%); Group IV, OQ559391 (98.14%); Group V, OQ520881 (99.31%), OQ553797 (99.64%); Group VI, OQ553801 (94.04%); Group VII, OQ521663 (99.82%); Group VIII, OQ521667 (97.21%), OQ520564 (99.65%); Group IX, OQ520337 (97.58%); Group X, OQ550975 (100%); Group XI, OQ520880 (95.76%), OQ553805 (98.76%), OQ559564 (98.57%), OQ521665 (99.48%). Query coverage ranged between 80 and 100%.

Spontaneous fermentation monitoring

Spontaneous fermentation of Tannat and Marselan grape musts was complete after 12 days. The results of the physicochemical analyses of these musts are shown in Tables 2 and 3.

Tannat grapes from the 2019 and 2021 vintages registered the highest initial total acidity (Table 2), whereas the lowest values were determined in the Marselan variety from the 2020 vintage (Table 3). At the end of the fermentation process, total acidity was slightly higher than that reported

Table 2 Physicochemical parameters of spontaneously fermented Tannat musts

Tannat variety						
Chemical parameter	2019		2020		2021	
	Initial	Final	Initial	Final	Initial	Final
Total soluble solids (%)	19.50 ± 0.71	8.28 ± 3.15	23.30 ± 0.20	7.60 ± 0.1	21.25 ± 0.07	9.75 ± 0.07
Total acidity (g tartaric acid /L)	6.42 ± 0.59	8.50 ± 0.24	4.37 ± 0.28	7.91 ± 0.1	6.67 ± 0.05	8.96 ± 0.08
Volatile acidity (g acetic acid/L)	–	0.30 ± 0.02	–	0,20 ± 0.01	–	0.31 ± 0.01
Total Carbon (%)	8.03 ± 0.3	5.21 ± 2.17	8.61 ± 0.04	5.42 ± 0.04	9.19 ± 0.02	6.15 ± 0.11
Alcohol concentration (% v/v)	–	7.19 ± 0.73	–	5.75 ± 1.58	–	7.17 ± 1.55
pH	3.43 ± 0.13	3.38 ± 0.09	3.66 ± 0.11	3.72 ± 0.15	3.66 ± 0.11	3.72 ± 0.15

Table 3 Physicochemical parameters of spontaneously fermented Marselan musts

Marselan variety						
Chemical parameter	2019		2020		2021	
	Initial	Final	Initial	Final	Initial	Final
Total soluble solids (%)	18.9 ± 1.70	7.58 ± 1.63	23.80 ± 0.1	7.70 ± 0.1	20.40 ± 0.14	9.25 ± 0.45
Total acidity (g tartaric acid/L)	5.09 ± 0.83	8.34 ± 1.89	4.09 ± 0.37	9.02 ± 0.10	4.55 ± 0.07	9.55 ± 0.07
Volatile acidity (g acetic acid/L)	–	0.38 ± 0.02	–	0.30 ± 0.00	–	0.30 ± 0.00
Total Carbon (%)	7.54 ± 0.59	5.34 ± 1.04	8.80 ± 0.26	5.90 ± 0.79	9.25 ± 0.45	6.51 ± 0.06
Alcohol concentration (% v/v)	–	7.45 ± 0.73	–	6.40 ± 3.78	–	9.74 ± 0.06
pH	3.53 ± 0.01	3.28 ± 0.04	3.52 ± 0.26	3.50 ± 0.14	4.04 ± 0.01	3.84 ± 0.07

in other studies (Franco-Bañuelos et al. 2017; Piccardo and Zamora 2021). Despite this increase, these values were equally low. For other varieties, some authors have informed a decrease in this parameter under similar conditions (Raymond Eder et al. 2017; Raymond Eder and Rosa 2019).

The volatile acidity of wines is constituted by 99% acetic acid. During alcoholic fermentation, fermentative yeasts produce variable quantities of volatile acidity, depending on the yeast strain, sugar content, and temperature of fermentation. Amounts from 0.2 to 0.8 g/L are acceptable but should not exceed 1.3 g/L (Cioch-Skoneczny et al. 2021). As shown in Tables 2 and 3, the registered values were within this range.

A constant reduction in total carbon concentration was observed at the end of each assay and was attributed to carbon dioxide loss during alcoholic fermentation. Likewise, ethanol concentration increased during the same period, indicating the advancement of the fermentative process. Final alcohol concentrations resulted similar to the values reported in vinifications carried out with musts in analogous physicochemical (initial total soluble solids) and environmental conditions (Raymond Eder et al. 2017; Raymond Eder and Rosa 2019).

The evolution of spontaneous fermentation of Tannat and Marselan musts is shown in Fig. 4a and b. In general, after 8 days of fermentation, no variation in total soluble solids was observed thus indicating the end of the process.

In the 2019 vintage, total soluble solids at harvest were 19°Bx for both grape varieties. This value results extremely low if the aim is to obtain wines with an alcoholic graduation greater than 10% v/v. According to information provided by Instituto Nacional de Tecnología Agropecuaria (2019), it was verified that during the period among November 2018 and January 2019, the monthly average rainfall was much higher than the historical measure. This hydrological excess increases fruit size; they develop more aqueous, with poor sugar content and richer in acids, which could cause a delay in ripening (Ramos and Martínez De Toda 2022; Veselá et al. 2022). Therefore, it can be assumed that excessive rainfall could be the reason for the lower total soluble solids content.

Yeast diversity during spontaneous fermentation of grape musts

The contribution of yeast species during the different stages (initial, middle, and final) of spontaneous fermentation of Marselan and Tannat grape musts is shown in Fig. 5 and 6. A great variability in species was observed at the beginning of the process, except for the Tannat and Marselan 2021

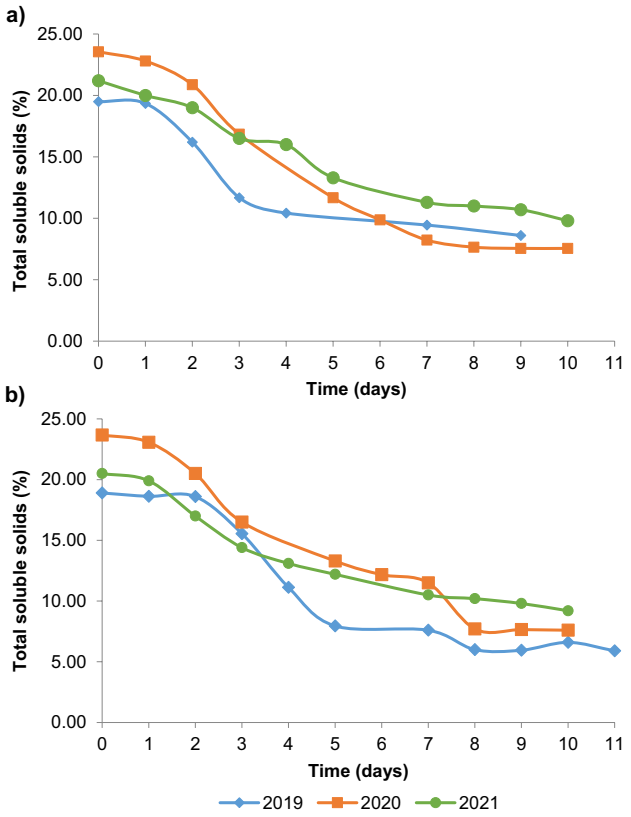

Fig. 4 Evolution of total soluble solids in spontaneously fermenting Tannat (**a**) and Marselan (**b**) musts during the 2019, 2020, and 2021 vintages

vintage. As alcoholic fermentation progressed, some species disappeared, and only those that could adapt to the new environmental conditions (higher ethanol content) remained viable (Albergaria and Arneborg 2016).

From the 2019 and 2021 vintages, *P. kudriavzevii* was the main non-*Saccharomyces* species coexisting with *S. cerevisiae* at advanced stages during Tannat fermentation. It was also found in 2020 vintage musts, but in a low number. *S. cerevisiae* was not isolated during fermentation of Marselan grapes from the 2019 vintage whereas *P. kudriavzevii* was the dominant species. In contrast, in the 2020 vintage, *S. cerevisiae* was the only species isolated during the final stages of fermentation. The results for both varieties differ from previous reports that identified *Aureobasidium*, *Hanseniaspora*, *Metschnikowia*, *Starmerella*, *Lachancea,* and *Candida* as the dominant non-*Saccharomyces* genera in grape musts from different wine regions, while the genus *Pichia* was less frequently identified (Maturano et al. 2016; Raymond Eder et al. 2017; Vaudano et al. 2019; Mateus et al. 2020).

Ethanol production varied in Tannat and Marselan spontaneous fermentations over the three studied vintages. Considering the 2020 vintage, *P. kudriavzevii* was not found in Marselan but appeared in low quantities in Tannat. As shown in Table 3, this situation corresponds to a lower ethanol content. On the other hand, the highest ethanol concentration (9% v/v) was determined in Marselan musts from the 2021 vintage. When analyzing the species present at the end of fermentation, *S. cerevisiae* and *P. kudriavzevii* were isolated in almost the same proportion. This could indicate their role in ethanol production. Kaur et al. (2019) studied a *P. kudriavzevii* isolated from fruits and reported their potential to

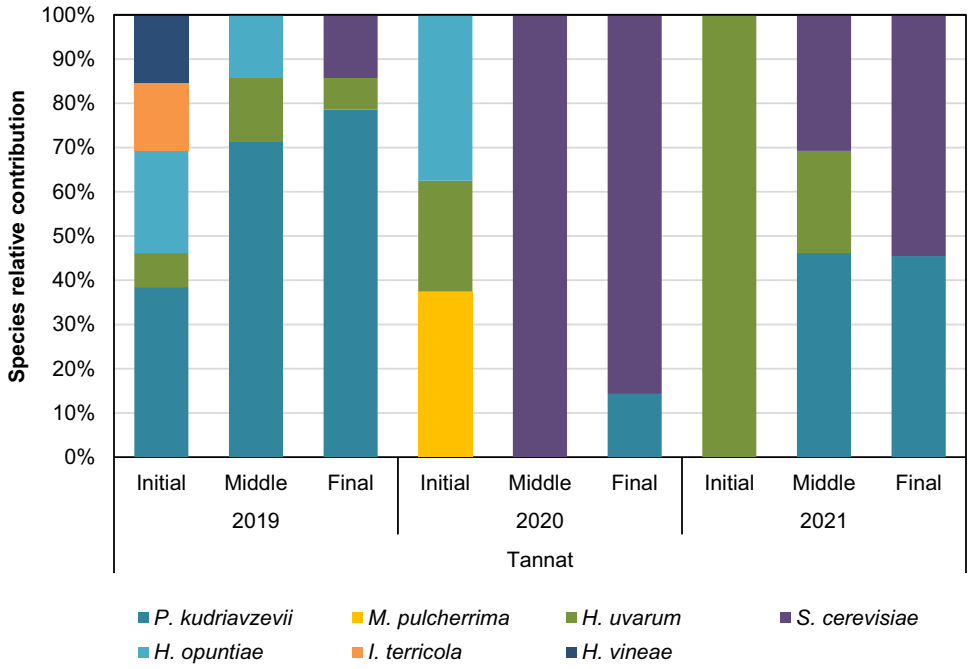

Fig. 5 Relative contribution of yeast species during spontaneous fermentation of Tannat grape musts at different stages (initial, middle and final)

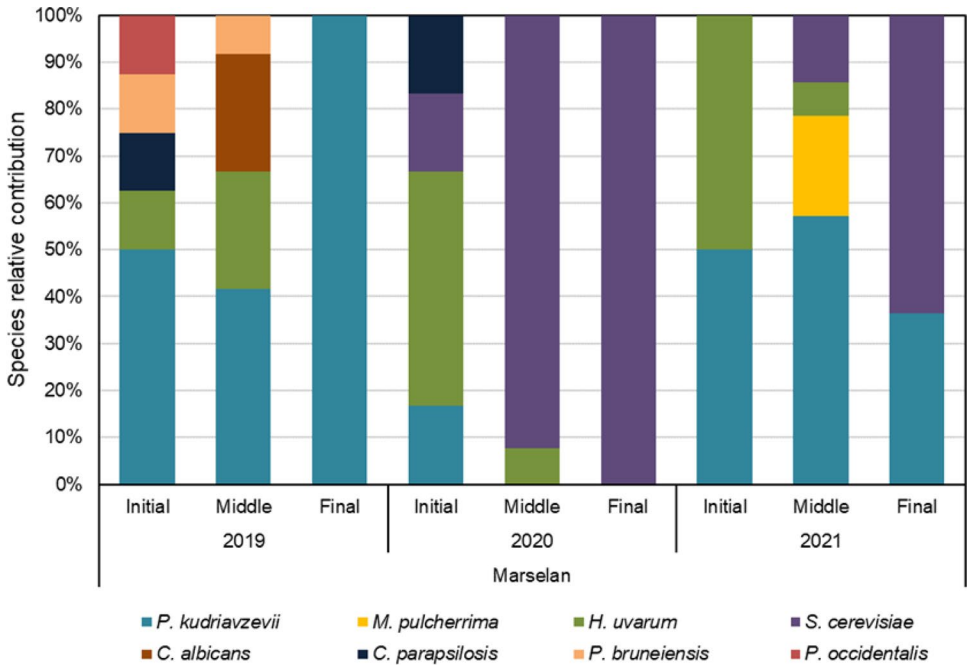

Fig. 6 Relative contribution of yeast species during spontaneous fermentation of Marselan grape musts at different stages (initial, middle, and final)

ferment sugars with ethanol production. In addition to high ethanol production, Nieto-Sarabia et al. (2022) showed that *P. kudriavzevii* had an ethanol tolerance superior to that of commercial *S. cerevisiae*. Aponte and Giuseppe (2016) reported that *P. kudriavzevii* isolated from Aglianico grapes produced 11% (v/v) ethanol.

In general, non-*Saccharomyces* yeasts have been reported to have lower fermentative capacities than *S. cerevisiae* (Polizotto et al. 2016). However, according to the results of this study (and others not shown), it can be stated that *P. kudriavzevii* could carry out grape musts fermentation with a good ethanol ratio.

In Argentina, *P. kudriavzevii* has been associated with spontaneous grape fermentation. One of these was isolated from the Isabella variety in the Córdoba province (Argentina) (Raymond Eder et al. 2017). In addition, del Mónaco et al. (2014) found it in Malbec grapes from Patagonia, Argentina, during the initial stages of spontaneous fermentation. In Zona Alta del Río Mendoza (Cuyo region), spontaneous fermentation of Malbec grapes has been studied, but the presence of this species has not been reported (Combina et al. 2005). On the other hand, Maturano et al. (2016) isolated it from Malbec grape must in a low proportion with respect to others. It is important to note that in all cases, the number of isolates was low, and *P. kudriavzevii* was not the main isolated species.

It is well known that *S. cerevisiae* belongs to the native grape microorganisms, it can be isolated from spontaneous fermentations and is responsible for the alcoholic fermentation during winemaking processes. However, the results found in Marselan grapes from the 2019 vintage showed that alcoholic fermentation was mainly carried out by *P. kudriavzevii* (Fig. 6). It can be seen that in middle and final stages over the three vintages (with the exception of Marselan 2020 because *P. kudriavzevii* was not isolated), *S. cerevisiae* and *P. kudriavzevii* were present, which indicated that spontaneous fermentation was carried out by both species, and sometimes *P. kudriavzevii* was the dominant one.

Conclusions

The yeast microbiota isolated and identified in this study, constitutes the first study of Tannat and Marselan varieties in Argentina. The evolution of *Saccharomyces* and non-*Saccharomyces* yeasts during must fermentation was investigated.

In addition, the morphologies of *H. opuntiae*, *P. bruneiensis,* and *P. occidentalis* on WL agar, which have not been previously reported, were described. Sequencing analysis confirmed the identification based on PCR–RFLP analysis. A similar diversity of yeast species was observed in both varieties.

In contrast, this work allowed the association of *P. kudriavzevii* (non-*Saccharomyces* yeast) with spontaneous fermentation of grape musts from the Concordia (Entre Ríos, Argentina) region. This species coexisted with *S. cerevisiae* at different stages of alcoholic fermentation. Moreover, *P. kudriavzevii* was the dominant species in some fermentations and produced good ethanol yield. Therefore, it can be concluded that this yeast species

exhibits a high potential for further exploration since it seems a good candidate to formulate a mixed culture with S. cerevisiae. In this sense, further research such as oenological characterization (sulfite tolerance, production of aroma compounds and biogenic amine) is essential to validate its role.

Acknowledgements The authors would like to thank Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) "Dr. Carlos G. Malbrán" (Argentina) for providing *S. cerevisiae* ATCC 9763. In addition, special thanks to Lic. Carolina Chacón for language assistance.

Author contributions Liliana Gerard made the design of the work, analysed, interpreted the obtained results and contributed to the manuscript writing and its final revision. She also developed the molecular identification of the isolated yeasts. María Belén Corrado and Carina Soldá performed the tracking of the grapes spontaneous fermentation, isolated and characterised yeasts species.They also managed activities to annotate (produce metadata), scrubbed data and maintained research data for initial and subsequent use. Cristina Davies managed and coordinated the responsibility for the activity planning and execution. She also made the manuscript writing, final critical revision and approval of the version to be published. María Gabriela Dalzotto contributed to the manuscript writing, editing and final revision. Sofía Esteche contributed to developing the molecular identification of the isolated yeasts. All authors reviewed the manuscript.

Funding This work was funded by Secretaría de Ciencia y Técnica, Universidad Nacional de Entre Ríos (Argentina), PID UNER N° 8109, Res. C.S. N° 186/19.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

References

Albergaria H, Arneborg N (2016) Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation processes: role of physiological fitness and microbial interactions. Appl Microbiol Biotechnol 100:2035–2046. https://doi.org/10.1007/s00253-015-7255-0

Aponte M, Giuseppe B (2016) Potential role of yeast strains isolated from grapes in the production of Taurasi DOCG front. Microbiol 1:809. https://doi.org/10.3389/fmicb.2016.00809

Barkhuizen JH, Coetzee G, van Rensburg E, Görgens JF (2021) The effect of growth rate on the production and vitality of non-*Saccharomyces* wine yeast in aerobic fed-batch culture. Bioprocess Biosyst Eng 44(12):2655–2665. https://doi.org/10.1007/s00449-021-02634-3

Cavazza A, Grando M, Zini C (1992) Rilevazione della flora microbica di mosti e vini. Dossier Biotecnologie 9:1–4

Cimini A, Moresi M (2022) Research trends in the oenological and viticulture sectors. Aust J Grape Wine Res 28(3):475–491. https://doi.org/10.1111/ajgw.12546

Cioch-Skoneczny M, Satora P, Skoneczny S, Klimczak K (2021) Physicochemical characterization of wines produced using indigenous yeasts from cold climate grapes. Eur Food Res Technol 247(1):201–209. https://doi.org/10.1007/s00217-020-03618-5

Combina M, Elía A, Mercado L, Catania C, Ganga A, Martinez C (2005) Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza. Argentina Int J Food Microbiol 99(3):237–243. https://doi.org/10.1016/j.ijfoodmicro.2004.08.017

Cray JA, Bell ANW, Bhaganna P, Mswaka AY, Timson DJ, Hallsworth JE (2013) The biology of habitat dominance; can microbes behave as weeds? Microb Biotechnol 6(5):453–492. https://doi.org/10.1111/1751-7915.12027

Del Fresno JM, Morata A, Loira I, Bañuelos MA, Escott C, Benito S, González Chamorro C, Suárez-Lepe JA (2017) Use of non-*Saccharomyces* in single-culture, mixed and sequential fermentation to improve red wine quality. Eur Food Res Technol 243(12):2175–2185. https://doi.org/10.1007/s00217-017-2920-4

del Mónaco SM, Barda NB, Rubio NC, Caballero AC (2014) Selection and characterization of a Patagonian *Pichia kudriavzevii* for wine deacidification. J Appl Microbiol 117(2):451–464. https://doi.org/10.1111/jam.12547

del Mónaco SM, Rodríguez ME, Lopes CA (2016) *Pichia kudriavzevii* as a representative yeast of North Patagonian winemaking terroir. Int J Food Microbiol 230:31–39. https://doi.org/10.1016/j.ijfoodmicro.2016.04.017

Drumonde-Neves J, Fernandes T, Lima T, Pais C, Franco-Duarte R (2021) Learning from 80 years of studies: A comprehensive catalogue of non-*Saccharomyces* yeasts associated with viticulture and winemaking. FEMS Yeast Res 21(3):1–3. https://doi.org/10.1093/femsyr/foab017

Franco-Bañuelos A, Contreras-Martínez CS, Carranza-Téllez J, Carranza-Concha J (2017) Total phenolic content and antioxidant capacity of non-native wine grapes grown in Zacatecas. Mexico Agrociencia 51(6):661–671

García-Béjar B, Árevalo-Villena M, Briones A (2021) Characterization of yeast population from unstudied natural sources in La Mancha region. J Appl Microbiol 130(3):650–664. https://doi.org/10.1111/jam.14795

Garofalo C, Russo P, Beneduce L, Massa S, Spano G, Capozzi V (2016) Non-*Saccharomyces* biodiversity in wine and the 'microbial terroir': a survey on Nero di Troia wine from the Apulian region. Italy Ann Microbiol 66:143–150. https://doi.org/10.1007/s13213-015-1090-5

Grangeteau C, Gerhards D, von Wallbrunn C, Alexandre H, Rousseaux S, Guilloux-Benatier M (2016) Persistence of two non-*Saccharomyces* yeasts (*Hanseniaspora* and *Starmerella*) in the cellar. Front Microbiol 7:268. https://doi.org/10.3389/fmicb.2016.00268

Guaragnella N, Chiara M, Capece A, Romano P, Pietrafesa R, Siesto G, Manzari C, Pesole G (2020) Genome sequencing and comparative analysis of three *Hanseniaspora uvarum* indigenous wine strains reveal remarkable biotechnological potential. Front Microbiol 10:1–14. https://doi.org/10.3389/fmicb.2019.03133

Instituto Nacional de Tecnología Agropecuaria (INTA) (2019) Informe sobre el efecto de exceso hídrico en los distintos cultivos en el área influencia de la EEA Concordia. Available from: https://inta.gob.ar/sites/default/files/inta-concordia_informe_exceso_hidrico_situacion_area_de_influencia_eea_inta_concordia_2019.pdf

Jolly NP, Varela C, Pretorius IS (2014) Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res 14(2):215–237. https://doi.org/10.1111/1567-1364.12111

Kaur S, Oberoi HS, Phutela R (2019) Isolation and characterization of a non-*Saccharomyces* yeast with improved functional characteristics for ethanol production. Int J Microbiol Res 26(4):42829. https://doi.org/10.9734/mrji/2018/v26i43007

Lai YT, Hsieh CW, Lo YC, Liou BK, Lin HW, Hou CY, Cheng KC (2022) Isolation and identification of aroma-producing non-*Saccharomyces* yeast strains and the enological characteristic comparison in wine making. LWT 154:112653. https://doi.org/10.1016/j.lwt.2021.112653

Li J, Hu W, Huang X, Xu Y (2018) Investigation of yeast population diversity and dynamics in spontaneous fermentation of Vidal blanc icewine by traditional culture-dependent and high-throughput sequencing methods. Food Res Int 112:66–77. https://doi.org/10.1016/j.foodres.2018.06.011

Liu L, Zhao PT, Hu CY, Tian D, Deng H, Meng YH (2022) Screening low-methanol and high-aroma produced yeasts for cider fermentation by transcriptive characterization. Front Microbiol 13:1042613. https://doi.org/10.3389/fmicb.2022.1042613

Martin V, Valera M, Medina K, Boido E, Carrau F (2018) Oenological impact of the *Hanseniaspora/Kloeckera* yeast genus on wines—a review. Ferment 4(3):76. https://doi.org/10.3390/fermentation4030076

Mateus D, Sousa S, Coimbra C, Rogerson FS, Simões J (2020) Identification and characterization of non-*Saccharomyces* species isolated from port wine spontaneous fermentations. Foods 9(2):120. https://doi.org/10.3390/foods9020120

Maturano YP, Mestre MV, Combina M, Toro ME, Vazquez F, Esteve-Zarzoso B (2016) Culture-dependent and independent techniques to monitor yeast species during cold soak carried out at different temperatures in winemaking. Int J Food Microbiol 21(237):142–149. https://doi.org/10.1016/j.ijfoodmicro.2016.08.013

Maturano YP, Mestre MV, Kuchen B, Toro ME, Mercado LA, Vazquez F, Combina M (2019) Optimization of fermentation-relevant factors: a strategy to reduce ethanol in red wine by sequential culture of native yeasts. Int J Food Microbiol 289:40–48. https://doi.org/10.1016/j.ijfoodmicro.2018.08.016

Mendoza LM, Vega-Lopez GA, Fernández de Ullivarri M, Raya RR (2019) Population and oenological characteristics of non-*Saccharomyces* yeasts associated with grapes of Northwestern Argentina. Arch Microbiol 201(2):235–244. https://doi.org/10.1007/s00203-018-1601-4

Mestre Furlani MV, Maturano YP, Combina M, Mercado LA, Toro ME, Vázquez F (2017) Selection of non-*Saccharomyces* yeasts to be used in grape musts with high alcoholic potential: a strategy to obtain wines with reduced ethanol content. FEMS Yeast Res 17(2):1–10. https://doi.org/10.1093/femsyr/fox010

Nieto-Sarabia VL, Ballinas-Cesatti CB, Melgar-Lalanne G, Cristiani-Urbina E, Morales-Barrera L (2022) Isolation, identification, and kinetic and thermodynamic characterization of a *Pichia kudriavzevii* yeast strain capable of fermentation. Food Bioprod Process 131:109–124. https://doi.org/10.1016/j.fbp.2021.10.013

Nisiotou AA, Spiropoulos AE, Nychas GJ (2007) Yeast community structures and dynamics in healthy and *Botrytis*-affected grape must fermentations. Appl Environ Microbiol 73(21):6705–6713. https://doi.org/10.1128/AEM.01279-07

O'Donnell K (1993) *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds) The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. CAB International, Wallingford, UK, pp 225–233

Official Methods of Analysis of AOAC INTERNATIONAL (2019) 21st Eds, AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 920.70.

OIV Compendium of International Methods of Wine and Must Analysis (2020) Volume 1, Paris, France: International Organisation of Vine and Wine

Padilla B, Gil JV, Manzanares P (2016) Past and future of non-*Saccharomyces* yeasts: from spoilage microorganisms to biotechnological tools for improving wine aroma complexity. Front Microbiol 7(411):1–20. https://doi.org/10.3389/fmicb.2016.00411

Pallmann CL, Brown JA, Olineka TL, Cocolin L, Mills DA, Bisson LF (2001) Use of WL medium to profile native flora fermentations. Am J Enol Vitic 52(3):198–203

Pham T, Wimalasena T, Box WG, Koivuranta K, Storgårds E, Smart KA, Gibson BR (2011) Evaluation of ITS PCR and RFLP for differentiation and identification of brewing yeast and brewery "wild" yeast contaminants. J Inst Brew 117(4):556–568. https://doi.org/10.1002/j.2050-0416.2011.tb00504.x

Piccardo D, Zamora F (2021) González-Neves G (2021) Desarrollo y evaluación de alternativas tecnológicas para reducir el contenido de alcohol y el pH de vinos tintos. Memoria Investig Ing 20:24–33. https://doi.org/10.36561/ing.20.4

Polizzotto G, Barone E, Ponticello G, Fasciana T, Barbera D, Corona O, Amore G, Giammanco A, Oliva D (2016) Isolation, identification and oenological characterization of non-*Saccharomyces* yeasts in a Mediterranean island. Lett Appl Microbiol 63(2):131–138. https://doi.org/10.1111/lam.12599

Ramos MC, Martínez De Toda F (2022) Influence of weather conditions and projected climate change scenarios on the suitability of *Vitis vinifera* cv. Carignan in Rioja DOCa. Spain Int J Biometeorol 66:1067–1078. https://doi.org/10.1007/s00484-022-02258-6

Raymond Eder ML, Rosa AL (2019) Yeast diversity in *Vitis* non-*vinifera* ecosystems. Rev Argent Microbiol 51(3):278–283. https://doi.org/10.1016/j.ram.2018.09.004

Raymond Eder ML, Reynoso C, Lauret SC, Rosa AL (2017) Isolation and Identification of the indigenous yeast population during spontaneous fermentation of Isabella (*Vitis labrusca* L.) grape must. Front Microbiol. https://doi.org/10.3389/fmicb.2017.00532

Shi WK, Wang J, Chen FS, Zhang XY (2019) Effect of *Issatchenkia terricola* and *Pichia kudriavzevii* on wine flavor and quality through simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae*. LWT. https://doi.org/10.1016/j.lwt.2019.108477

Sipiczki M (2012) *Pichia bruneiensis* sp. nov., a biofilm-producing dimorphic yeast species isolated from flowers in Borneo. Int J Syst Evol Microbiol 62:3099–3104. https://doi.org/10.1099/ijs.0.044974-0

Sumby KM, Caliani NS, Jiranek V (2021) Yeast diversity in the vineyard: how it is defined, measured and influenced by fungicides. Aust J Grape Wine Res 27(2):169–193. https://doi.org/10.1111/ajgw.12479

Varela C, Borneman AR (2017) Yeasts found in vineyards and wineries. Yeast 34(3):111–128. https://doi.org/10.1002/yea.3219

Vaudano E, Quinterno G, Costantini A, Pulcini L, Pessione E, Garcia-Moruno E (2019) Yeast distribution in Grignolino grapes growing in a new vineyard in Piedmont and the technological characterization of indigenous *Saccharomyces* spp. strains. Int J Food Microbiol 289:154–161. https://doi.org/10.1016/j.ijfoodmicro.2018.09.016

Veselá K, Severová L, Svoboda R (2022) The impact of temperature and precipitation change on the production of grapes in the Czech Republic. Sustainability. https://doi.org/10.3390/su14063202

Wang C, Liu Y (2013) Dynamic study of yeast species and *Saccharomyces cerevisiae* strains during the spontaneous fermentations of Muscat blanc in Jingyang. China Food Microbiol 33(2):172–177. https://doi.org/10.1016/j.fm.2012.09.014

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, New York, NY, USA, pp 315–22. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Wilson K (2001) Preparation of genomic DNA from bacteria. Curr Protoc Mol Biol 56:241–245. https://doi.org/10.1002/0471142727.mb0204s56

Zabukovec P, Čadež N, Čuš F (2020) Isolation and identification of indigenous wine yeasts and their use in alcoholic fermentation. Food Technol Biotechnol 58(3):337–347. https://doi.org/10.17113/ftb.58.03.20.6677

Zhang J, Plowman JE, Tian B, Clerens S, On SLW (2021) Application of MALDI-TOF analysis to reveal diversity and dynamics of winemaking yeast species in wild-fermented, organically produced,

New Zealand Pinot Noir wine. Food Microbiol. 99:103824. https://doi.org/10.1016/j.fm.2021.103824