International Journal of Food Microbiology 305 (2019) 108255

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

Occurrence and enological properties of two new non-conventional yeasts (*Nakazawaea ishiwadae* and *Lodderomyces elongisporus*) in wine fermentations

Javier Ruiz[a,1], Nora Ortega[a,1], María Martín-Santamaría[a], Alberto Acedo[b], Domingo Marquina[a], Olga Pascual[c], Nicolas Rozès[c], Fernando Zamora[c], Antonio Santos[a], Ignacio Belda[b,d,*]

[a] Department of Genetics, Physiology and Microbiology, Unit of Microbiology, Biology Faculty, Complutense University of Madrid, 28040 Madrid, Spain
[b] Science Department, Biome Makers Spain, 47011 Valladolid, Spain
[c] Departament de Bioquímica i Biotecnologia, Facultat d'Enologia de Tarragona, Universitat Rovira i Virgili, C/Marcel.li Domingo s/n, 43007 Tarragona, Spain
[d] Department of Biology, Geology, Physics & Inorganic Chemistry, Area of Biodiversity and Conservation, Rey Juan Carlos University, 28933 Móstoles, Spain

ARTICLE INFO

Keywords:
Wine microbiome
Wine yeasts
Yeast metabolism
Non-*Saccharomyces* yeasts
Lodderomyces elongisporus
Nakazawaea ishiwadae

ABSTRACT

The microbial diversity of wine alcoholic fermentation is not restricted to the presence and activity of *Saccharomyces* yeast strains. Some non-*Saccharomyces* species have been described as part of the fermentative microbiota, specially found in the initial steps of wine fermentations. These species may play roles from wine spoilage to flavor quality enhancement. From a large number of wine fermentations (429 wine samples), analyzed by ITS-amplicon sequencing to define their mycobiome, 2 non-conventional yeast species (*Nakazawaea ishiwadae* and *Lodderomyces elongisporus*) were detected, in a very limited number of samples but in significant levels of relative abundance.

One strain of each species was isolated and their technological and enological potential have been characterized in this work. Compared with the *Saccharomyces cerevisiae* Viniferm Revelacion wine strain, the studied *N. ishiwadae* BMK17.1 and *L. elongisporus* BMK12.5 strains showed, as expected, a lower tolerance and growth fitness in high ethanol concentrations. However, *N. ishiwadae* BMK17.1 was able to grow also at 15% ethanol and *L. elongisporus* BMK12.5 at 10% reaching, in the latter case, slightly higher efficiency rates than *S. cerevisiae* at this level. Contrary to most non-*Saccharomyces* yeasts, these species were able to growth in presence of high doses of potassium-metabisulfite, reaching in both cases higher efficiency rates than *S. cerevisiae*. A notable affinity of *L. elongisporus* BMK12.5 for high pH values was clearly observed.

Their fermentation kinetics and the final chemical-analytical characterization were studied in micro-fermentation assays, using synthetic grape must. *L. elongisporus* BMK12.5 was able to complete, in single inoculation, the sugar fermentation after 19 days, but, *N. ishiwadae* BMK17.1 left about 80 g/L sugars at this time. Co-inoculation assays (in a 1:100 proportion of *S. cerevisiae*:non-*Saccharomyces* strains) finished sugar consumption with similar kinetics than the *S. cerevisiae* single inoculation, in the case of *L. elongisporus* BMK12.5 co-inoculation, and with lower kinetics when using *N. ishiwadae* BMK17.1. A remarkable malic acid consumption and a low acetic acid production was associated with *L. elongisporus* BMK12.5 fermentations, together with a high production of 3-methyl-1-butanol and 2-phenylethanol, and the release of high amounts of proteins into the wines. *N. ishiwadae* BMK17.1, although unable to finish the fermentation itself, showed a high production of oligosaccharides and volatile compounds such as isobutanol or isobutyric acid.

This work reports, for the first time, the occurrence and enological potential of two strains pertaining to the non-conventional yeast genera *Lodderomyces* and *Nakazawaea*.

* Corresponding author at: Department of Biology, Geology, Physics & Inorganic Chemistry, Area of Biodiversity and Conservation, Rey Juan Carlos University, 28933 Móstoles, Spain.
E-mail address: ignacio.belda@urjc.es (I. Belda).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ijfoodmicro.2019.108255
Received 12 March 2019; Received in revised form 7 May 2019; Accepted 19 June 2019
Available online 20 June 2019

1. Introduction

The use of metagenomics in wine science has revealed a microbial diversity higher than expected both in the vineyard and in the winery (reviewed in Belda et al., 2017a). The microbial complexity of wine fermentations is key to understanding the qualitative determinants when turning a grape must into wine (Bokulich et al., 2016). From spoilage species to wine enhancers, a great diversity of taxa, and thus metabolisms (metabolic routes and regulation patterns), have attracted microbiologists to the field of wine science.

Some decades ago, 'non-*Saccharomyces*' was a term usually associated with wine spoilage; mainly due to increases in volatile acidity (due to the presence of certain apiculate yeasts) or, more particularly, with the production of non-desired compounds such as volatile phenols (*i.e.* from *Brettanomyces* species) (Amerine and Cruess, 1960; Le Roux et al., 1973; Rankine, 1972; Van der Walt and Van Kerken, 1958; Van Zyl and Du Plessis, 1961). In the last two decades, a large number of studies reported the metabolic/enzymatic potential of certain non-*Saccharomyces* yeasts and their potential role for improving some technological and sensorial aspects of wine (reviewed in Balmaseda et al., 2018; Belda et al., 2017b; Ciani et al., 2006; Jolly et al., 2014). Thus, the deliberate use of non-*Saccharomyces* yeasts as biological tools (inoculum) in wine fermentations has become a trend in modern wine industry. Nowadays, several strains of non-*Saccharomyces* species are produced commercially (mainly pertaining to *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima* and *Pichia kluyveri* species). These species are used in winemaking with objectives such as: i) increasing the varietal aroma fraction of wines (Belda et al., 2017c; Ruiz et al., 2018; Sadoudi et al., 2012); ii) controlling wine acidity (Gobbi et al., 2013); iii) improving color extraction and mouthfeel properties (Belda et al., 2016a; Lleixà et al., 2016); iv) reducing the ethanol content of wines (Contreras et al., 2014); and, more recently, v) to improve foaming properties of sparkling wines (Medina-Trujillo et al., 2017a).

During the last years, the number of scientific works exploring the use of the above-mentioned species for understanding their enological potential has increased. However, none or at most very few new species has been explored apart from those already reported in the initial works or in classical reviews in the field of more than a decade ago. These include *Candida*, *Debaryomyces*, *Hanseniaspora/Kloeckera*, *Hansenula*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* species (Ciani et al., 2006; Esteve-Zarzoso et al., 1998).

The first works using Next Generation Sequencing (NGS) technology to explore the complexity of wine fermentations were carried out by Bokulich et al. (2012) and Bokulich and Mills (2013) for bacterial and fungal communities, respectively. For mycobiome studies, several papers have demonstrated the great diversity of yeast species in wine fermentations and its dependence with vineyard *terroir* aspects and the enological practices applied in the winery (Bokulich et al., 2014, 2016; Grangeteau et al., 2017; Portillo and Mas, 2016). Nevertheless, the microbial diversity detected by NGS analysis is rarely confirmed by culture-dependent methods, and even less by physiological characterization after isolation.

In this work, we reported the incidence in wine samples of two non-*Saccharomyces* yeast species, pertaining to non-conventional genus for wine environment (*Nakazawaea ishiwadae* and *Lodderomyces elongisporus*). Although periodically mentioned in scientific literature associated with wine-related environments, there is no information about their enological properties. One strain of each species was isolated from wine samples, and, after a basic characterization of some technological properties, the enological potential of the mentioned strains was studied in laboratory scale wine fermentations. After this first report, it is necessary to increase the number of *N. ishiwadae* and *L. elongisporus* wine strains for further genomic, phenotypic and enological studies for the understanding of their potential intra-specific diversity.

2. Material and methods

2.1. Survey of *N. ishiwadae* and *L. elongisporus* occurrence in wine yeast populations

The occurrence and relative abundance of *N. ishiwadae* and *L. elongisporus* in wines were determined from an extensive survey of 429 wine samples (coming from different winemaking stages, as is summarized in Table S1, detailed in Table S2). The mycobiome profile of the 429 wines was analyzed by Next Generation Sequencing ITS-amplicon metabarcoding, using WineSeq technology (patent number: Patent WO2017096385), in a MiSeq® Sequencer (Illumina Inc., San Diego, Ca, USA). Considering the total fungal populations of the wine samples analyzed, a positive occurrence was considered when the relative abundance of the studied species was higher than 0.01%. The information about occurrence and relative abundance is showed in Fig. 1 and Table S1.

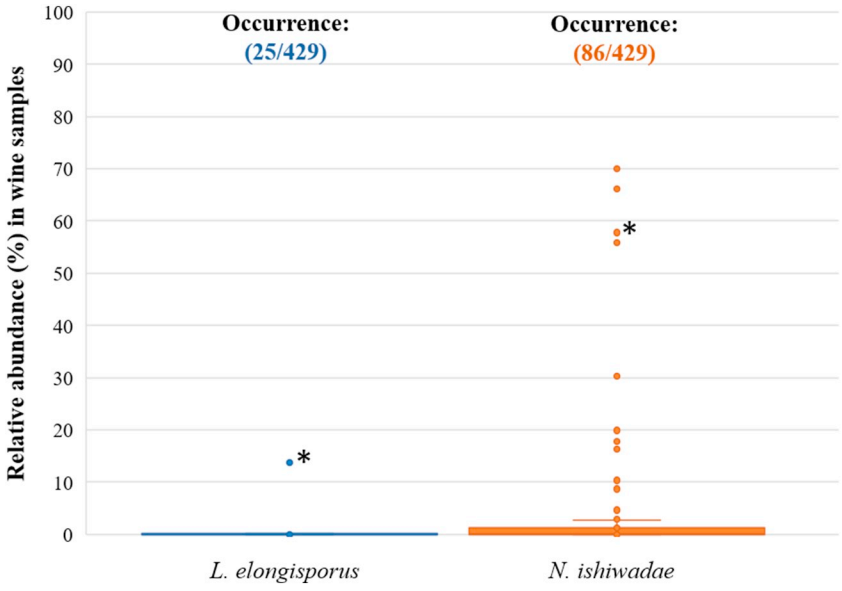

Fig. 1. Relative abundance (%) of *Lodderomyces elongisporus* and *Nakazawaea ishiwadae* yeasts in wine samples mycobiomes. Occurrence has been counted when relative abundance of a species is > 0.01%. Data obtained from a survey of 429 wine samples (a basic description of the origin (wine fermentation stage) of the samples where these yeasts were detected is included in Table S1). * symbol near a dot indicates the specific wine sample from where the studied strains (*L. elongisporus* BMK12.5 and *N. ishiwadae* BMK17.1) were isolated.

2.2. Yeast strains and molecular identification

S. cerevisiae Viniferm Revelacion strain used in this study was provided by Agrovin S.A. (Alcazar de San Juan, Spain). *N. ishiwadae* BMK17.1 (GenBank accession number: MK610797) and *L. elongisporus* BMK12.5 (GenBank accession number: MK610798) strains were isolated from spontaneous red wine fermentations of Tempranillo grapes from Ribera del Duero Appellation of Origin (Spain) (see Table S2 for further details). *S. cerevisiae* Hansen BY4741 strain was used as the sensitive control for the killer assay, confirming that none of the two new isolates (*N. ishiwadae* BMK17.1 and *L. elongisporus* BMK12.5) showed killer activity against this strain (that is sensitive to K2 killer toxin).

After isolation, the two new non-*Saccharomyces* isolates were identified by sequencing of the 26S large subunit of rRNA gene. Total DNA was extracted following the procedure described by Querol et al. (1992) and the mentioned 26S region for sequencing was amplified using an Eppendorf Mastercycler apparatus as described by Kurtzman and Robnett (1997) with forward NL-1 primer (5′-GCATATCAATAAGCGG AGGAAAAG-3′) and reverse NL-4 primer (5′-GGTCCG TGTTTCAAGA CGG-3′). Sequences obtained were compared and identified by BLAST-search (GenBank; www.ncbi.nlm.nih.gov).

It is necessary to mention that the two studied strains (*N. ishiwadae* BMK17. and *L. elongisporus* BMK12.5) were isolated in the framework of a global metagenomic study (using only culture-independent technology). Plate culturing approaches (using Sabouraud glucose agar (PanReac, Barcelona, Spain), at a growth temperature of 28 °C for 48 h) were occasionally used to confirm the presence of rare species originally detected by NGS techniques. Thus, the specific samples from where the two studied strains were casually isolated are highlighted in Fig. 1 and Table S2.

2.3. Phenotypic characterization

Some basic technological properties of *N. ishiwadae* BMK17.1 and *L. elongisporus* BMK12.5 were tested using *S. cerevisiae* Viniferm Revelacion as a control. Ethanol, sulfite and high osmolarity tolerances, and growth ability at different pH were tested. Growing assays were performed by triplicate on 12-well plates. Strains were inoculated, at final cell concentration of 10^6 cells/mL, in 2 mL of Synthetic Medium (SM) (yeast nitrogen base (Difco) 0.17%, glucose 2%, pH 3.5) after 24 h of preculture on the same medium. Different medium compositions were setting depending on the trait tested. Ethanol tolerance was tested by measuring the yeast growth on SM under three different ethanol concentrations (5%, 10% and 15%). Likewise, sulfite resistance was measured using three different sodium metabisulfite (MBS) concentrations (100, 200 and 400 ppm). High osmolarity tolerance was assayed in SM medium changing the initial glucose concentration to 20% of glucose. SM medium adjusted to three different pH values (3.5, 3.8 and 4) was used to test the yeast growth under different pH conditions. Assays were performed at 28 °C under orbital shaking at 120 rpm. Optical density (600 nm) was measured at different time points (0 h, 16 h, 24 h, 48 h and 90 h), using a microplate reader Varioskan Flash Multimode Reader (Thermo Scientific). Growth rate and efficiency (total variation in cell density) were extracted from growth curves by using *GrowthRates* R package (Hall et al., 2014), adjusting the growth curves to a Baranyi model (Baranyi and Roberts, 1994).

Killer activity was assayed as described by Santos et al. (2009). Yeasts were inoculated in a ~1 cm diameter zone onto plates with YMA-MB medium (1% glucose, 0.3% yeast extract, 0.3% malt extract and 0.5% proteose peptone no. 3, supplemented with 30 mg/L of methylene blue, 3% NaCl and 2% agar) previously seeded with a lawn of the sensitive yeast (*S. cerevisiae* Hansen BY4741). The plates were incubated at 20 °C for a week. Killer yeasts were identified by a clear zone of inhibition surrounding them.

2.4. Micro-vinifications and growth kinetics

Fermentations were carried out at micro-vinification scale using Synthetic Grape Must (SGM) medium described by Henschke and Jiranek (1993), with some modifications regarding nitrogen content. The yeast assimilable nitrogen (YAN) in the synthetic must was 750 mg N L^{-1}; divided in 26 mg N L^{-1} of ammonia-nitrogen ($(NH_4)_2HPO_4$) and 724 mg N L^{-1} of amino acids, maintaining the amino acids proportion of the original recipe. Musts were prepared and sterilized by 0.45 μm filtration (Thermo Scientific™ Nalgene™ Rapid-Flow™). Precultures were performed in SGM medium, and inocula were adjusted to reach a final cell concentration of 10^6 cells/mL by determining O.D. at 600 nm. Fermentation assays were performed by triplicate in 100 mL flasks containing 90 mL of SGM. Flasks were provided with an air-lock system allowing CO_2 to escape and sampling under sterile conditions. Fermentations were performed at 20 °C under orbital shaking at 100 rpm. Magnetic stirring was used to homogenize the medium before sampling.

Five different assays were carried out: 1) inoculation with *S. cerevisiae* Viniferm Revelacion (Sc); 2) inoculation with *N. ishiwadae* BMK17.1 (Ni); 3) inoculation with *L. elongisporus* BMK12.5 (Le); 4) mixed inoculation with *S. cerevisiae* Viniferm Revelacion and *N. ishiwadae* BMK17.1 (Sc + Ni), in 1:100 ratio, and 5) inoculation with *S. cerevisiae* Viniferm Revelacion and *L. elongisporus* BMK12.5 (Sc + Le), in 1:100 ratio.

Cell concentrations were followed by plating 50 μL of the appropriate dilution on Sabouraud glucose agar (PanReac, Barcelona, Spain) plates, for total yeast counts, and on lysine medium (Oxoid, Hampshire, UK), for non-*Saccharomyces* counts. Colonies were counted after 48 h at 28 °C. Due to the invasive growth of the non-*Saccharomyces* yeasts, a precise cell population monitoring was not always possible. Nevertheless, following this procedure, the time when non-*Saccharomyces* yeasts disappeared from the fermentation was estimated. Fermentation kinetics were monitored by measuring weight loss every 24 h. Fermentations were estimated to be finished when the weight loss was < 0.01 g per day. Once fermentations were finished, cultures were centrifugated at 7000 rpm for 10 min to remove biomass. Then, supernatants were stored at −20 °C until further analysis.

2.5. Basic wine parameters quantification

To quantify basic parameters of finished fermentations, here we used the Fourier Transform Infrared Spectroscopy method, using a monochromator instrument OenoFoss™ (Hilleroed, Denmark; technical specifications at: https://www.fossanalytics.com/en/products/oenofoss). The following parameters were determined by this method: ethanol content, total acidity, pH, volatile acidity, malic acid, lactic acid (that was not detected in any samples analyzed), glucose and fructose.

2.6. Analysis of wine aroma compounds

The procedure of extraction and separation of wine aroma compounds used in this work was a modification of the method by Ortega et al. (2001). Briefly, to 10 mL of wine 2.5 g of $(NH_4)_2SO_4$, 40 μL of a standard solution mixture (4-methyl-2-pentanol, 800 mg/L and heptanoic acid, 700 mg/L dissolved in ethanol) and 0.4 mL of dichloromethane (PanReac, Barcelona, Spain) were added.

The samples were agitated at 60 rpm for 90 min at room temperature and then centrifuged for 10 min at 5000 rpm. The upper aqueous phase was discarded and the dichloromethane lower phase was transferred to a GC-vial. The extract (3 μL) was analyzed in split mode (5:1, 30 mL/min) by an Agilent GC 6850 (Agilent Technologies, Böblingen, Germany) coupled to a flame ionization detector. The volatile compounds were separated on a HP-FFAP column (30 m × 0.25 mm, 0.25 μm, Agilent). The oven temperature was initially held at 35 °C for 5 min, raised by 7 °C/min to 100 °C and finally raised by 3 °C/min to

220 °C for 2 min. The temperature of the injector and detector are 220 and 250 °C respectively. The flow of helium carrier gas was 1.1 mL/min. Volatile compounds were identified and quantified by comparison with standards dissolved in a synthetic wine (12% (v/v) ethanol, 5 g/L of tartaric acid and pH adjusted to 3.5 with 1 M sodium hydroxide) and analyzed following the same procedure. All the standards were purchased from Sigma-Aldrich. Supplementary Table S3 indicates all the analyzed volatile compounds and their retention time.

2.7. Polysaccharide extraction and determination by HRSEC-RID

The samples were processed using the methodology described by Ayestarán et al. (2004). Briefly, 10-mL samples in duplicate were concentrated to a final volume of 2 mL using a vacuum evaporator (Univap 148 100ECH; Progen Scientific, United Kingdom). Total soluble polysaccharides were precipitated by adding 10 mL of cold acidified ethanol (hydrochloric acid 0.3 M in absolute ethanol) and kept at 4 °C for 24 h. The samples were then centrifuged (10,000 ×*g* for 15 min), and the supernatants were discarded. Finally, the precipitates were dissolved in 1 mL of ultrapure water, frozen to −20 °C, and freeze-dried (Telstar LyoQuest HT40).

The soluble fractions were analyzed by high-resolution size exclusion chromatography (HRSEC) to determine molecular distribution and quantify the polysaccharides obtained from the samples (Ayestarán et al., 2004). The lyophilized samples were resuspended in 1 mL of 50 mM ammonium formate and filtered through 0.22-μm acetate cellulose filters (Merck Millipore), and 100 μL were injected into the chromatographic system. The analyses were carried out in an HPLC Agilent 1200 Series system (Agilent Technologies Inc.) with a refractive index detector (RID). Separation was carried out at 20 °C using two Shodex gel permeation HPLC columns (OHpak SB-803 HQ and SB-804 HQ, 300 mm × 8 mm i.d.; Showa Denko). The mobile phase consisted of an aqueous solution of 50 mM ammonium format applied with a constant flow of 0.6 mL/min for 60 min and a cell RID temperature of 35 °C.

The molecular weight (MW) distribution of the wine fractions was followed by calibration with a Shodex P-82 pullulan calibration kit (P-5, MW = 5.9 kDa; P-10, MW = 11.8 kDa; P-20, MW = 22.8 kDa; P-50, MW = 47.5 kDa; P-100, MW = 112 kDa; P-200, MW = 212 kDa; P-400, MW = 404 kDa; and P-800, MW = 788 kDa) purchased from Waters and four dextrans (BioChemika; 12, 25, 50 and 80 kDa) purchased from Fluka. The polysaccharides were quantified according to the peak area for each fraction using the external standard method with pectin and dextran commercial standards (Sigma-Aldrich) in a range between 0 and 2 g/L ($r^2 > 0.99$).

2.8. Determination of proteins by HRSEC-DAD

The samples were processed using the methodology described by Canals et al. (1998). Briefly, 15 mL of each sample was dialyzed in duplicate in tubes with an MW cutoff of 3.5 kDa (Membrane Filtration Products Inc.). The dialyzed samples were lyophilized and preserved at −20 °C.

Proteins were analyzed by HRSEC to determine molecular distribution and quantify the proteins obtained from the samples (Canals et al., 1998). The lyophilized samples were resuspended in 0.6 μL of 300 mmol/L ammonium acetate and centrifuged (12,000 ×*g* for 5 min). The supernatant was filtered through 0.22-μm acetate cellulose filters (Merck Millipore), and 100 μL of supernatant was injected into the chromatographic system. The analyses were performed in an HPLC Agilent 1200 Series system (Agilent Technologies) with a diode array detector (DAD) to monitor output at 230 and 320 nm. Separation was carried out at 20 °C using an S 165 Shodex gel permeation HPLC column (OHpak 166 SB-803 HQ, 300 mm × 8 mm i.d.; Showa Denko). The mobile phase consisted of an aqueous solution of 300 mmol/L ammonium acetate applied at a constant flow of 0.6 mL/min for 70 min. The proteins were quantified according to the peak area for each fraction using the external standard method with bovine serum albumin (Sigma-Aldrich) in a range between 0 and 1 mg/mL ($r^2 > 0.99$).

3. Results and discussion

3.1. Occurrence and abundance N. ishiwadae and L. elongisporus in wine

Fig. 1 shows that both *N. ishiwadae* and *L. elongisporus* are rare species in wine fermentations. Based on a survey of 429 wine fermentation samples, taken from different fermentation stages, only 5.83% and 20.05% of the samples showed *L. elongisporus* and *N. ishiwadae*, respectively, in relative abundance values higher than 0.01% (Table S1). It is notable that both species could be detected throughout the wine production process, from grape juice to wine under maturation. It is especially notable that *N. ishiwadae* could be found in the advanced stages of the winemaking process (Table S1), where non-*Saccharomyces* yeast are generally not isolated due to the harsh environment. Although enologically relevant, this information should be taken with caution since the use of DNA sequencing technologies to detect the presence of microbial species did not guarantee the viability and metabolic activity of the species detected. When detected, their mean relative abundance values are of 0.64% and 4.99% for *L. elongisporus* and *N. ishiwadae*, respectively. Thus, the current scarcity of works on the enological role of these species is not strange. A few papers report the presence of these species in wine-related environments: *L. elongisporus* in a grape cluster (Parish and Carroll, 1985), and in a wine filler (Malfeito-Ferreira et al., 1997); and *N. ishiwadae* from wine barrels (Guzzon et al., 2011) and its cleaning waste water (Portugal et al., 2015), and from wine fermentations (Boynton and Greig, 2016; Cioch-Skoneczny et al., 2018). However, the incidence and abundance patterns of these species in a large set of samples have not been reported until now.

3.2. Technological properties of N. ishiwadae BMK17.1 and L. elongisporus BMK12.5 strains

For a basic understanding on the metabolic properties of *N. ishiwadae* and *L. elongisporus* we measured growth parameters (growth rate and efficiency) under some wine-related conditions (tolerance to ethanol and metabisulfite, and growth ability at grape must osmolarity conditions and pH values) in micro-cultivations assays (Table 1). *L. elongisporus* showed in most cases the lowest growth rates. However, despite the presence of ethanol, in all other conditions assayed, *L. elongisporus* BMK12.5 showed the highest efficiency values when comparing with *N. ishiwadae* BMK17.1 and the *S. cerevisiae* control strain. Especially interesting is the case of *L. elongisporus* behavior at 5% and 10% of ethanol where the growth rates were significantly lower, but the final efficiency was similar at 5% ethanol and even slightly higher at 10% ethanol concentration than in the control *S. cerevisiae*. Although *L. elongisporus* BMK12.5 was not able to grow at 15% ethanol concentration, *N. ishiwadae* BMK17.1 did, but with a very limited growth rate value. Contrary to what was expected, both *L. elongisporus* and *N. ishiwadae* strains were able to grow in presence of MBS, even at very high doses (400 ppm). Here, *L. elongisporus* BMK12.5 stands out because of its growth efficiency, reaching the highest values when compared with *N. ishiwadae* and even *S. cerevisiae*. In terms of growth rate, none of the species were affected by the increasing concentrations of MBS. Finally, regarding pH effect on yeast growth, *N. ishiwadae* and *S. cerevisiae* did not show a significant variation on their behavior at pH from 3 to 4. However, a subtle preference of *L. elongisporus* for the higher pH values can be perceived. This is an interesting result since, as stated by Mira de Orduña (2010), the generalized increase in wine musts pH, as a consequence of global warming, can lead to significant changes in the microbial ecology of musts and wines, increasing the risk of spoilage and organoleptic degradation. Therefore, this yeast must to be under consideration, due to the possible increase in its occurrence under these

Table 1
Growth rate (G. rate) and efficiency of the studied yeast strains under different wine-related *in vitro* conditions.

Assay	Measure	*S. cerevisiae* Vin. Revelacion	*L. elongisporus* BMK12.5	*N. ishiwadae* BMK17.1	p-Value
Ethanol 5%	G. rate	0.53 ± 0.07c	0.05 ± 0.03a	0.20 ± 0.05b	***
	Efficiency	1.68 ± 0.01c	1.39 ± 0.04a	1.50 ± 0.03b	***
Ethanol 10%	G. rate	0.38 ± 0.01b	0.10 ± 0.04a	0.34 ± 0.01b	***
	Efficiency	1.69 ± 0.01b	1.73 ± 0.09b	1.33 ± 0.15a	**
Ethanol 15%	G. rate	0.13 ± 0.03c	0.00 ± 0.00a	0.07 ± 0.02b	***
	Efficiency	1.45 ± 0.13c	0.00 ± 0.00a	0.30 ± 0.05b	***
Glucose 20%	G. rate	0.30 ± 0.01c	0.20 ± 0.01b	0.11 ± 0.01a	***
	Efficiency	1.22 ± 0.18b	1.30 ± 0.01b	0.92 ± 0.02a	*
MBS 100	G. rate	0.31 ± 0.01c	0.22 ± 0.05b	0.14 ± 0.01a	**
	Efficiency	1.14 ± 0.00a	1.26 ± 0.06b	1.17 ± 0.02a	*
MBS 200	G. rate	0.32 ± 0.06b	0.24 ± 0.03b	0.11 ± 0.00a	**
	Efficiency	1.13 ± 0.04a	1.40 ± 0.05b	1.13 ± 0.02a	***
MBS 400	G. rate	0.29 ± 0.01c	0.23 ± 0.03b	0.11 ± 0.01a	***
	Efficiency	1.05 ± 0.08a	1.43 ± 0.01c	1.20 ± 0.04b	***
pH 3.5	G. rate	0.21 ± 0.01b	0.25 ± 0.00c	0.12 ± 0.02a	***
	Efficiency	1.07 ± 0.03a	1.39 ± 0.16b	1.17 ± 0.02a	*
pH 3.8	G. rate	0.23 ± 0.00b	0.23 ± 0.00b	0.11 ± 0.00a	***
	Efficiency	1.06 ± 0.02a	1.53 ± 0.02c	1.16 ± 0.02b	***
pH 4.0	G. rate	0.22 ± 0.00b	0.24 ± 0.02b	0.12 ± 0.01a	***
	Efficiency	1.10 ± 0.10a	1.59 ± 0.10b	1.12 ± 0.01a	***

All data are expressed as the average values of 3 replicates ± standard deviation. Different letters indicate the existence of statistical differences (*p < 0.05; **p < 0.01; ***p < 0.005) between the different yeasts analyzed.

new conditions.

Regarding the capacity of killer toxins production, neither *N. ishiwadae* BMK17.1 nor *L. elongisporus* BMK12.5 showed killer activity against *S. cerevisiae* killer sensitive strain (Hansen BY4741).

3.3. Fermentation assays and enological properties of N. ishiwadae BMK17.1 and L. elongisporus BMK12.5 strains

Using synthetic grape must, the main aim of this work was to investigate the fermentation behavior and the potential contribution to wine chemical composition of the studied non-*Saccharomyces* strains. For that, a co-inoculation assay was performed with a 1:100 ratio (*S. cerevisiae*:non-*Saccharomyces* strain) to simulate the natural under-representation of *S. cerevisiae* yeasts in grape musts, and the correspondent single inoculation assays with *L. elongisporus*, *N. ishiwadae* and *S. cerevisiae*. Some scientific works used to follow sequential inoculation strategies when studying complex fermentations with non-*Saccharomyces* and *S. cerevisiae* yeasts. This is especially relevant when trying to maximize the action time of non-*Saccharomyces* strains in the process (Belda et al., 2015; Gobbi et al., 2013). However, here we decide to perform a simultaneous co-inoculation strategy, aiming to understand the natural behavior of these species in their co-existence with *S. cerevisiae* in grape must, and not as a potential industrial inoculum. Table 2 shows that *N. ishiwadae* in single inoculation was unable to complete the entire fermentation, leaving more than the 40% of initial glucose and fructose concentration. It is also noticeable that also, when using *S. cerevisiae* in co-inoculation with *N. ishiwadae*, the fermentation process was not fully completed. This observation should be remarked in the light of the results of Boynton and Greig (2016) where they defined *N. ishiwadae* as a keystone species in wine fermentations. In ecological terms, it means that, as occurred with *S. cerevisiae* (that is also defined as a keystone species in wine fermentation environment), these species are able to dominate, or at least to determine, the microbial dynamics of wine fermentations also when starting the process at low abundances values. In our study, the co-existence of these two species (*N. ishiwadae* and *S. cerevisiae*) in the experimental conditions can imply a competitive environment that substantially modifies the metabolic performance of the two species. Although this point should be further studied in future yeast-yeast interactions assays, here we should highlight the concentration of acetic acid (by-product of the primary metabolism of yeasts) observed in Ni + Sc assay (Table 2). It is significantly higher than any other value. As a by-product of the primary metabolism of yeasts, acetic acid is an interesting marker of yeasts' performance in wine fermentations, and its notable increase in Ni + Sc assay can be originated by the above-mentioned yeast-yeast interactions and its eco-physiological consequences. On the other hand, *L. elongisporus* was able to complete the fermentation process both in single and in co-inoculation assays. In accordance to the growth characteristics observed in Table 1, *L. elongisporus* followed a slow fermentation kinetic, but was able to deplete all the sugars after 19 days of fermentation (Fig. 2). Although not statistically significant, a slight reduction in final ethanol content can be seen when using *L. elongisporus*. This fact is of great importance since lower ethanol production yields of some non-*Saccharomyces* yeasts is in the spotlight of wine research as a biological alternative to reduce the alcohol content of wines (Ciani et al., 2016). However, the most remarkable contribution of *L.*

Table 2
Analytical results of the main enological parameters at the end of the alcoholic fermentation.

Assay	Residual sugars (g/L)	Ethanol (%)	Titratable acidity (g/L)	pH	Volatile acidity (g/L)	Malic acid (g/L)	Fructose (g/L)	Glucose (g/L)
Sc	1.00 ± 0.14a	10.85 ± 0.07b	6.80 ± 0.14c	3.25 ± 0.01ab	0.78 ± 0.09c	3.50 ± 0.14bc	0.45 ± 0.05a	1.55 ± 0.05b
Sc + Le	2.27 ± 1.50a	10.27 ± 0.51b	6.27 ± 0.21b	3.27 ± 0.01ab	0.72 ± 0.02bc	3.13 ± 0.21b	1.77 ± 1.50a	1.67 ± 0.06bc
Sc + Ni	11.05 ± 3.85b	10.30 ± 0.42b	6.80 ± 0.00c	3.02 ± 0.23a	1.02 ± 0.13d	3.55 ± 0.21bc	10.50 ± 3.60b	1.70 ± 0.00c
Le	1.63 ± 0.55a	10.43 ± 0.75b	4.83 ± 0.21a	3.38 ± 0.02b	0.39 ± 0.02a	2.40 ± 0.10a	1.27 ± 0.38a	1.23 ± 0.06a
Ni	80.93 ± 6.63c	7.23 ± 0.21a	4.93 ± 0.21a	3.28 ± 0.01a.b	0.58 ± 0.01b	4.93 ± 0.21d	N/A	N/A
p-Value	***	***	***	*	***	***	***	***

All data are expressed as the average values of 3 replicates ± standard deviation. Different letters indicate the existence of statistical differences (*p < 0.05; ***p < 0.005) between the different fermentation assays (Sc: *Saccharomyces cerevisiae*; Lc: *Lodderomyces elongisporus*; Ni: *Nakazawaea ishiwadae*; Sc + Le: co-inoculation (1:100) of *S. cerevisiae* + *L. elongisporus*; Sc + Ni: co-inoculation (1:100) of *S. cerevisiae* + *N. ishiwadae*).

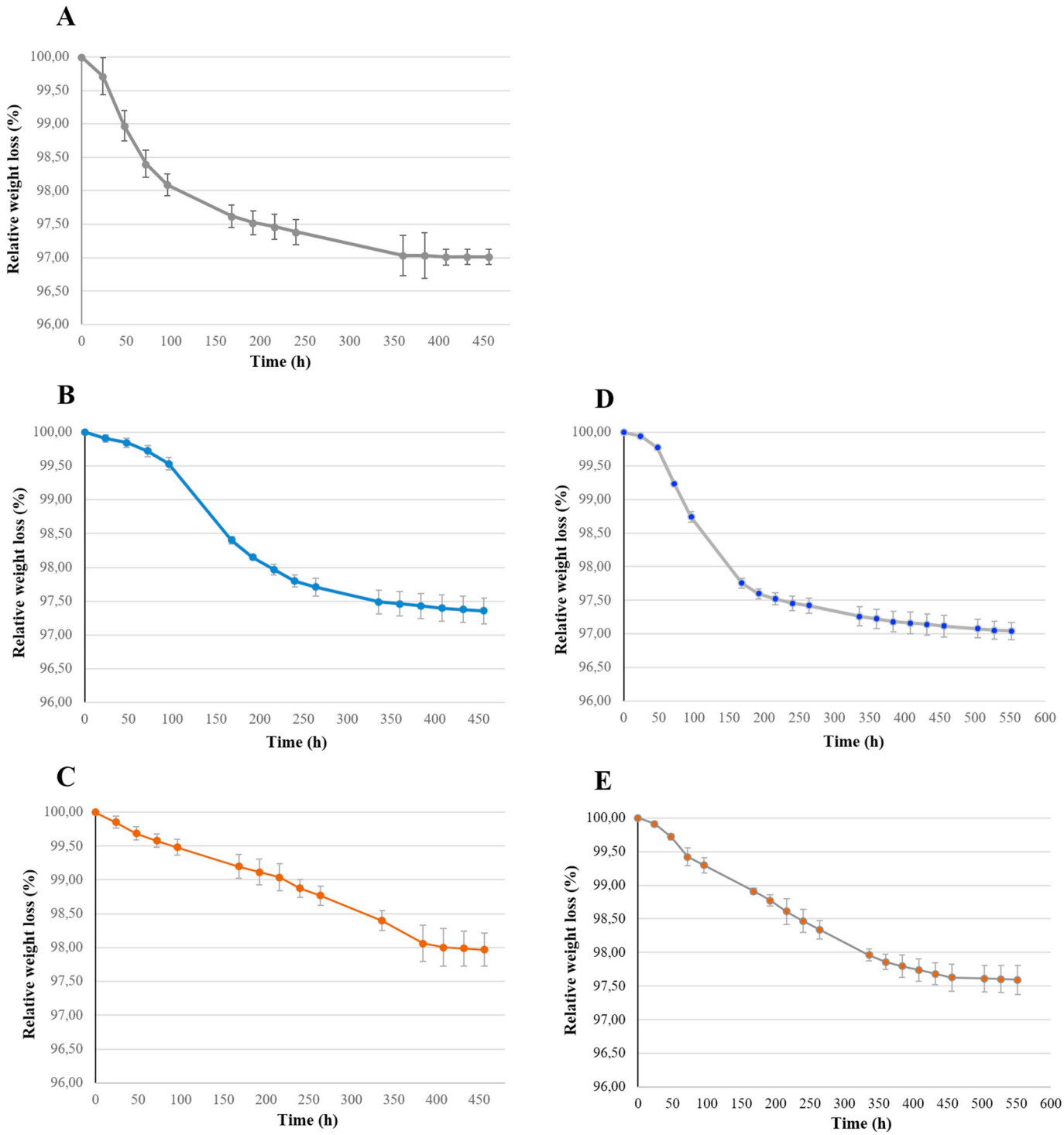

Fig. 2. Fermentation kinetics measured as relative weight loss in the different assays. A) Sc assay (*Saccharomyces cerevisiae* single inoculation); B) Le assay (*Lodderomyces elongisporus* single inoculation); C) Ni assay (*Nakazawaea ishiwadae* single inoculation); D) Sc + Le assay (co-inoculation (1:100) of *S. cerevisiae* + *L. elongisporus*); E) Sc + Ni assay (co-inoculation (1:100) of *S. cerevisiae* + *N. ishiwadae*).

elongisporus to the basic analytical profile of the wines was in the production of low levels of titratable and volatile acidity. These aspects are more significant when *L. elongisporus* is used as single inoculum, but a slight reduction in the levels of these parameters is observed also in co-inoculation with *S. cerevisiae*. In the same line, other non-*Saccharomyces* species, such as *T. delbrueckii*, have been reported as producers of low to moderate concentrations of acetic acid in wines (Belda et al., 2015; Bely et al., 2008; Renault et al., 2009). Contrary to that, in the co-inoculation assay Sc + Ni, the volatile acidity was increased, and the final pH was decreased compared with the control assay solely inoculated with *S. cerevisiae* Viniferm Revelacion (Sc) and the mixed inoculation with *L. elongisporus* (Sc + Le). As it has been extensively reported in the literature, a high production of acetic acid is one of the main characteristics justifying the initial consideration of most of non-*Saccharomyces* species a potential sources of wine spoilage (Jolly et al., 2014), but in this case, as stated before it seems to be an effect of the yeast-yeast interaction in the competitive environment established in Sc + Ni assay. Besides, the final content of malic acid was significantly reduced

Table 3
Volatile compounds (mg/L) at the end of the alcoholic fermentation.

Compound (mg/L)	Sc			Sc + Le			Sc + Ni			Le			Ni		
1-Propanol	18.82	±	2.28a	15.56	±	1.98a		n.d.			n.d.			n.d.	
2-Methyl-propanol	10.34	±	0.62a	12.27	±	1.08ab	16.8	±	2.90b	13.24	±	1.29ab	33.64	±	7.76c
3-Methyl-1-butanol acetate	0.60	±	0.15a	0.68	±	0.09a	0.77	±	0.11a			n.d.	0.75	±	0.09a
3-Methyl-1-butanol	45.30	±	4.07a	53.5	±	0.43b	46.75	±	5.61ab	70.05	±	1.64c		n.d.	
1-Pentanol		n.d.		0.14	±	0.24a	0.34	±	0.05a		n.d.			n.d.	
1-Hexanol	0.38	±	0.33a	0.59	±	0.19a	0.56	±	0.22a		n.d.		0.42	±	0.08a
2-Phenylethanol	7.47	±	2.35a	7.27	±	0.13a	12.98	±	2.57b	29.11	±	3.63c	13.42	±	2.59b
ΣHigher alcohols	82.91	±	9.8b	90.01	±	4.14b	78.2	±	11.46b	112.4	±	6.56d	48.23	±	10.52a
Isobutyl acetate	0.70	±	0.05b	0.49	±	0.01a	0.50	±	0.10a		n.d.		0.46	±	0.08a
Ethyl butyrate		n.d.			n.d.		2.42	±	0.59b	3.21	±	0.68b	1.00	±	0.31a
Ethyl lactate		n.d.		6.33	±	0.20a	6.88	±	0.59a		n.d.			n.d.	
2-Phenylethanol acetate		n.d.		1.93	±	0.28a		n.d.			n.d.			n.d.	
Ethyl dodecanoate		n.d.		0.72	±	0.06a		n.d.			n.d.			n.d.	
ΣEsters	0.70	±	0.05a	9.47	±	0.55d	9.80	±	1.28d	3.21	±	0.68c	1.46	±	0.39b
Propionic acid	7.64	±	1.99b	6.28	±	0.82b	3.91	±	0.88a		n.d.			n.d.	
Isobutyric acid		n.d.			n.d.		5.55	±	0.05a		n.d.		24.47	±	5.50b
Butyric acid	4.02	±	0.63b	3.34	±	0.97ab	1.89	±	0.51a		n.d.			n.d.	
3-Methyl-butanoic acid		n.d.			n.d.		1.68	±	0.88a		n.d.			n.d.	
Valeric acid	0.49	±	0.07a		n.d.			n.d.			n.d.			n.d.	
Hexanoic acid		n.d.			n.d.		2.78	±	0.40a		n.d.			n.d.	
Octanoic acid	0.71	±	0.28a	1.36	±	0.14b	0.33	±	0.05a	1.24	±	0.17b		n.d.	
Decanoic acid		n.d.		1.29	±	0.62a		n.d.			n.d.			n.d.	
Dodecanoic acid		n.d.			n.d.			n.d.		0.58	±	0.53a		n.d.	
ΣAcids	12.86	±	2.97b	12.27	±	2.55b	16.14	±	2.77bc	1.82	±	0.70a	24.47	±	5.50c

All data are expressed as the average values of 3 replicates ± standard deviation. Different letters indicate the existence of statistical differences ($p < 0.05$) between the different fermentation assays (Sc: *Saccharomyces cerevisiae*; Lc: *Lodderomyces elongisporus*; Ni: *Nakazawaea ishiwadae*; Sc + Le: co-inoculation (1:100) of *S. cerevisiae* + *L. elongisporus*; Sc + Ni: co-inoculation (1:100) of *S. cerevisiae* + *N. ishiwadae*).

when *L. elongisporus* participates in the fermentation. The ability of consuming malic acid (and other organic acids, such as gluconic acid) has been previously reported for *Schizosaccharomyces* species (Benito et al., 2013; Gao and Fleet, 1995).

Regarding the volatile fraction of the wines obtained, we can highlight the following species-associated patterns. The use of *L. elongisporus* BMK12.5 as single inoculum increased the concentration of aroma impact compounds such as higher alcohols, especially 3-methyl-1-butanol and 2-phenylethanol, impacting on fermented and floral aromas, respectively, compared with the single *S. cerevisiae* inoculation assay. When used in co-inoculation with *S. cerevisiae*, *L. elongisporus* BMK12.5 also resulted in increased 3-methyl-1-butanol concentration and production of the acetate ester of 2-phenylethanol (2-phenylethyl acetate), which contributes to the fruity notes of wine aroma. The increased production of 2-phenylethanol and 2-phenylethyl acetate in wine has been previously studied in *Hanseniaspora vineae* strains (Giorello et al., 2018; Lleixà et al., 2016). In contrast, *L. elongisporus* produced a significant lower concentration of acids compared with the single *S. cerevisiae* inoculation assay. The contribution of *N. ishiwadae*, as stated before, could not be adequately studied from its single inoculation, since it was unable to finish the fermentation. However, from Sc + Ni assay, and using Ni data as support, we observed a great ability of this strain to produce isobutanol, isobutyric acid and, as showed by *L. elongisporus*, the rose-like aroma 2-phenylethanol (Table 3).

Finally, Fig. 3 shows the final content of polysaccharides, oligosaccharides and proteins in the wines from the different assays. Here, the final protein concentration when *L. elongisporus* was used should be highlighted (both in Le and Sc + Le assays, but more remarkably in the former). This trait is of enological interest as proteins play an important role in wine stability, being the source of wine haze (Van Sluyter et al., 2015). However, protein haze is produced by proteins coming from the grape (thaumatin like proteins and chitinases) and not from the yeasts (Esteruelas et al., 2009). On the other hand, it has been reported that proteins play an important role in the stabilization of the foam of sparkling wines (Medina-Trujillo et al., 2017b) so this higher contribution of proteins could be positive for certain wine types. Apart from that, the concentration of oligosaccharides in the fermentation assay inoculated only with *N. ishiwadae* was notably higher than in all the other experiments. The presence in significant amounts of complex free oligosaccharides in red and white wines has been demonstrated before as they are of great interest due to its biological activity as prebiotic components of food (Bordiga et al., 2012), and in the specific case of wine, some oligosaccharides have physicochemical properties such as an ability to chelate cations (Cescutti and Rizzo, 2001) and could have a positive impact on the perception of astringency in wine (Quijada-Morín et al., 2014). However, as stated before, results from this experiment (Ni) should be taken with care, since they came from an unfinished fermentation.

4. Conclusions

The growing number of scientific works using NGS technology to explore the microbial complexity of wine fermentations should be the basis for discovering the role of non-conventional species in wine ecosystem and the final flavor properties of wines. In this work, a first enological characterization has been performed for 2 strains of the non-conventional yeasts *N. ishiwadae* and *L. elongisporus* species and reporting some interesting features, starting from their ability to grow in winemaking conditions (under high ethanol and MBS concentrations). The subtle affinity of *L. elongisporus* for high wine pH values (pH 4) is an interesting case to consider in the near future where one of the main effects of climate change in wine is the continuous increase of wine pH. This fact, associated with certain trends in wine industry, such as performing spontaneous fermentations and reducing the use of MBS can lead to the emergence of new microbial actors in winemaking. The dominance of *S. cerevisiae* should still be studied, but the entire wine microbial consortium should be taken into account by following ecological and microbiological approaches. Regarding organoleptic properties, both non-*Saccharomyces* species showed an impact in final wines. *L. elongisporus* fermentations achieved high concentrations of 3-methyl-1-butanol and 2-phenylethanol, and high amounts of proteins to the wines. Meanwhile *N. ishiwadae* showed a high release of oligosaccharides and volatile compounds such as isobutyric acid and isobutanol.

As stated before, here we present a first characterization of some

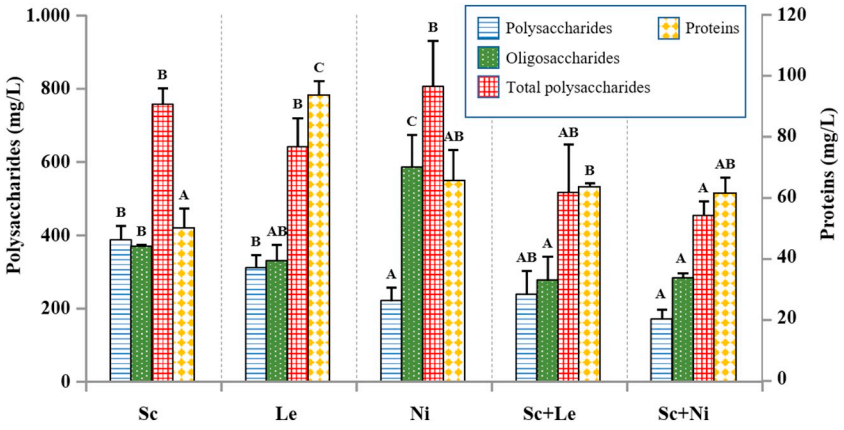

Fig. 3. Concentration of polysaccharides (blue-lines). oligosaccharides (green-dots). Sum of polysaccharides + oligosaccharides (red-squares) and proteins (yellow-diamonds) in the final wines obtained in the different assays (Sc: *Saccharomyces cerevisiae*; Lc: *Lodderomyces elongisporus*; Ni: *Nakazawaea ishiwadae*; Sc + Le: co-inoculation (1:100) of *S. cerevisiae* + *L. elongisporus*; Sc + Ni: co-inoculation (1:100) of *S. cerevisiae* + *N. ishiwadae*). All data are expressed as the average values of 3 replicates ± standard deviation. Different letters indicate the existence of statistical differences ($p < 0.05$) between the different fermentation conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

physiological and enological properties of two *N. ishiwadae* and *L. elongisporus* strains. However, as reported for all wine yeast species, there is a great intra-specific diversity in terms of wine-related metabolic properties (Belda et al., 2016b). Thus, after this first work, an effort of the wine microbiology research community is required for establishing a representative collection of strains from these species for a broader definition of their enological potential.

Declaration of Competing Interest

Alberto Acedo is an employee of Biome Makers and Ignacio Belda developed part of this work as employee of Biome Makers (granted by Spanish Ministry of Economy, Industry and Competitiveness).

Acknowledgements

Funding for the research in this paper was provided by Biome Makers Spain S.L. under the framework of the MINECO-financed project IDI-20180120 (CDTI, Spanish Ministry of Economy, Industry and Competitiveness, Spain). Javier Ruiz acknowledges to the Complutense University of Madrid for his doctoral grant (CT17/17-CT18/17) and Ignacio Belda acknowledges for his postdoctoral grant (PTQ-16-08253) provided by the Spanish Ministry of Economy, Industry and Competitiveness.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108255.

References

Amerine, M.A., Cruess, W.V., 1960. The Technology of Winemaking. The AVI Publishing Company, Inc., Connecticut.
Ayestarán, B., Guadalupe, Z., León, D., 2004. Quantification of major grape polysaccharides (*Tempranillo* V.) released by maceration enzymes during the fermentation process. Anal. Chim. Acta 513, 29–39.
Balmaseda, A., Bordons, A., Reguant, C., Bautista-Gallego, J., 2018. Non-*Saccharomyces* in wine: effect upon *Oenococcus oeni* and malolactic fermentation. Front. Microbiol. 9, 534. https://doi.org/10.3389/fmicb.2018.00534.
Baranyi, J., Roberts, T.A., 1994. A dynamic approach to predicting bacterial growth in food. Int. J. Food Microbiol. 23, 277–294.
Belda, I., Navascués, E., Marquina, D., Santos, A., Calderon, F., Benito, S., 2015. Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality. Appl. Microbiol. Biotechnol. 99, 1911–1922.
Belda, I., Conchillo, L.B., Ruiz, J., Navascués, E., Marquina, D., Santos, A., 2016a. Selection and use of pectinolytic yeasts for improving clarification and phenolic extraction in winemaking. Int. J. Food Microbiol. 257, 183–191.
Belda, I., Ruiz, J., Alastruey-Izquierdo, A., Navascués, E., Marquina, D., Santos, A., 2016b. Unraveling the enzymatic basis of wine "flavorome": a phylo-functional study of wine related yeast species. Front. Microbiol. 7, 12. https://doi.org/10.3389/fmicb.2016.00012.
Belda, I., Zarraonaindia, I., Perisin, M., Palacios, A., Acedo, A., 2017a. From vineyard soil to wine fermentation: microbiome approximations to explain the "terroir" concept. Front. Microbiol. 8, 821. https://doi.org/10.3389/fmicb.2017.00821.
Belda, I., Ruiz, J., Esteban-Fernández, A., Navascués, E., Marquina, D., Santos, A., Moreno-Arribas, M.V., 2017b. Microbial contribution to wine aroma and its intended use for wine quality improvement. Molecules 22, 189.
Belda, I., Ruiz, J., Beisert, B., Navascués, E., Marquina, D., Calderón, F., Rauhut, D., Benito, S., Santos, A., 2017c. Influence of *Torulaspora delbrueckii* in varietal thiol (3-SH and 4-MSP) release in wine sequential fermentations. Int. J. Food Microbiol. 223, 1–8.
Bely, M., Stoeckle, P., Masneuf-Pomarède, I., Dubourdieu, D., 2008. Impact of mixed *Torulaspora delbrueckii-Saccharomyces cerevisiae* culture on high-sugar fermentation. Int. J. Food Microbiol. 122, 312–320.
Benito, S., Palomero, F., Morata, A., Calderon, F., Palmero, D., Suarez-Lepe, J.A., 2013. Physiological features of *Schizosaccharomyces pombe* of interest in making of white wines. Eur. Food Res. Technol. 236, 29–36.
Bokulich, N.A., Mills, D.A., 2013. Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. Appl. Environ. Microbiol. 79 (8), 2519–2526.
Bokulich, N.A., Joseph, C.L., Allen, G., Benson, A.K., Mills, D.A., 2012. Next-generation sequencing reveals significant bacterial diversity of botrytized wine. PLoS One 7 (5), e36357. https://doi.org/10.1371/journal.pone.0036357.
Bokulich, N.A., Thorngate, J.H., Richardson, P.M., Mills, D.A., 2014. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc. Natl. Acad. Sci. U. S. A. 111 (1), E139–E148. https://doi.org/10.1073/pnas.1317377110.
Bokulich, N.A., Collins, T.S., Masarweh, C., Allen, G., Heymann, H., Ebeler, S.E., Mills, D.A., 2016. Associations among wine grape microbiome, metabolome, and fermentation behavior suggest microbial contribution to regional wine characteristics. mBio 7 (3), e00631-00616. https://doi.org/10.1128/mBio.00631-16.
Bordiga, M., Travaglia, F., Meyrand, M., German, J.B., Lebrilla, C.B., Coïsson, J.D., Arlorio, M., Barile, D., 2012. Identification and characterization of complex bioactive oligosaccharides in white and red wine by a combination of mass spectrometry and gas chromatography. J. Agric. Food Chem. 60, 3700–3707.
Boynton, P.J., Greig, D., 2016. Species richness influences wine ecosystem function through a dominant species. Fungal Ecol. 22, 61–72.
Canals, J.M., Arola, L., Zamora, F., 1998. Protein fraction analysis of white wine by FPLC. Am. J. Enol. Vitic. 49, 383–388.
Cescutti, P., Rizzo, R., 2001. Divalent cation interactions with oligogalacturonides. J. Agric. Food Chem. 49, 3262–3267.
Ciani, M., Beco, L., Comitini, F., 2006. Fermentation behavior and metabolic interactions of multistarter wine yeast fermentations. Int. J. Food Microbiol. 108, 239–245.
Ciani, M., Morales, P., Comitini, F., Tronchoni, J., Canonico, L., Curiel, J.A., Oro, L., Rodrigues, A.J., Gonzalez, R., 2016. Non-conventional yeast species for lowering ethanol content of wines. Front. Microbiol. 7, 642. https://doi.org/10.3389/fmicb.2016.00642.
Cioch-Skoneczny, M., Satora, P., Skotniczny, M., Skoneczny, S., 2018. Quantitative and qualitative composition of yeast microbiota in spontaneously fermented grape musts obtained from cool climate grape varieties 'Rondo' and 'Regent'. FEMS Yeast Res. 18, foy089. https://doi.org/10.1093/femsyr/foy089.
Contreras, A., Hidalgo, C., Henschke, P.A., Chambers, P.J., Curtin, C., Varela, C., 2014. Evaluation of non–*Saccharomyces* yeasts for the reduction of alcohol content in wine. Appl. Environ. Microbiol. 80, 1670–1678.
Esteruelas, M., Poinsaut, P., Sieczkowski, N., Manteau, S., Fort, F., Canals, J.M., Zamora, F., 2009. Characterization of natural haze protein in Sauvignon white wine. Food Chem. 113, 28–35.
Esteve-Zarzoso, B., Manzanares, P., Ramon, D., Querol, A., 1998. The role of non-*Saccharomyces* yeasts in industrial winemaking. Int. Microbiol. 1, 143–148.
Gao, C., Fleet, G.H., 1995. Degradation of malic and tartaric acids by high density cell suspensions of wine yeasts. Food Microbiol. 12, 65–71.
Giorello, F., Valera, M.J., Martin, V., Parada, A., Salzman, V., Camesasca, L., Fariña, L., Boido, E., Medina, K., Dellacassa, E., Berna, L., Aguilar, P.S., Mas, A., Gaggero, C., Carrau, F., 2018. Genomic and transcriptomic basis of *Hanseniaspora vineae*'s impact on flavor diversity and wine quality. Appl. Environ. Microbiol. e01959-18. https://doi.org/10.1128/AEM.01959-18.
Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2013. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and

sequential co–fermentation: a strategy to enhance acidity and improve the overall quality of wine. Food Microbiol. 33, 271–281.
Grangeteau, C., Roullier-Gall, C., Rousseaux, S., Gougeon, R.D., Schmitt-Kopplin, P., Alexandre, H., Guilloux-Benatier, M., 2017. Wine microbiology is driven by vineyard and winery anthropogenic factors. Microb. Biotechnol. 10 (2), 354–370.
Guzzon, R., Widmann, G., Malacarne, M., Nardin, T., Nicolini, G., Larcher, R., 2011. Survey of the yeast population inside wine barrels and the effects of certain techniques in preventing microbiological spoilage. Eur. Food Res. Technol. 233 (2), 285–291.
Hall, B.G., Acar, H., Nandipati, A., Barlow, M., 2014. Growth rates made easy. Mol. Biol. Evol. 31, 232–238.
Henschke, P.A., Jiranek, V., 1993. Yeasts-metabolism of nitrogen compounds. In: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology. Harwood Academic, Switzerland, pp. 77–164.
Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res. 14, 215–237.
Kurtzman, C.P., Robnett, C.J., 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. 35, 1216–1223.
Le Roux, G., Eschenbruch, R., de Bruin, S.I., 1973. The microbiology of South African wine-making. Part VIII. The microflora of healthy and *Botrytis cinerea* infected grapes. Phytophylactica 5, 51–54.
Lleixà, J., Martín, V., Portillo, M.d.C., Carrau, F., Beltran, G., Mas, A., 2016. Comparison of fermentation and wines produced by inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*. Front. Microbiol. 7, 338. https://doi.org/10.3389/fmicb.2016.00338.
Malfeito-Ferreira, M., Tareco, M., Loureiro, V., 1997. Fatty acid profiling: a feasible typing system to trace yeast contamination in wine bottling plants. Int. J. Food Microbiol. 38, 143–155.
Medina-Trujillo, L., González-Royo, E., Sieczkowski, N., Heras, J., Canals, J.M., Zamora, F., 2017a. Effect of sequential inoculation (*Torulaspora delbrueckii/Saccharomyces cerevisiae*) in the first fermentation on the foaming properties of sparkling wine. Eur. Food Res. Technol. 243, 681–688.
Medina-Trujillo, L., Matias-Guiu, P., López-Bonillo, F., Miquel Canals, J.M., Zamora, F., 2017b. Physicochemical characterization of the foam of white and rosé base wines for sparkling wine production (AOC Cava). Am. J. Enol. Vitic. 68, 485–495.
Mira de Orduña, R., 2010. Climate change associated effects on grape and wine quality and production. Foodserv. Res. Int. 43, 1844–1855.
Ortega, C., López, R., Cacho, J., Ferreira, V., 2001. Fast analysis of important wine volatile compounds development and validation of a new method based on gas chromatographic-flame ionisation detection analysis of dichloromethane microextracts. J. Chromatogr. A 923, 205–214.
Parish, M.E., Carroll, D.E., 1985. Indigenous yeasts associated with muscadine (*Vitis rotundifolia*) grapes and musts. Am. J. Enol. Vitic. 36 (2), 165–169.
Portillo, M.del., Mas, A., 2016. Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing. Food Sci. Technol. LEB. 72, 317–321.
Portugal, C., Pinto, L., Ribeiro, M., Tenorio, C., Igrejas, G., Ruiz-Larrea, F., 2015. Potential spoilage yeasts in winery environments: characterization and proteomic analysis of *Trigonopsis cantarellii*. Int. J. Food Microbiol. 210, 113–120.
Querol, A., Barrio, E., Huerta, T., Ramón, D., 1992. Molecular monitoring of wine fermentations conducted by active dry yeast strains. Appl. Environ. Microbiol. 58, 2948–2953.
Quijada-Morín, N., Williams, P., Rivas-Gonzalo, J.C., Doco, T., Escribano-Bailón, M.T., 2014. Polyphenolic, polysaccharide and oligosaccharide composition of Tempranillo red wines and their relationship with the perceived astringency. Food Chem. 154, 44–51.
Rankine, B.C., 1972. Influence of yeast strain and malo-lactic fermentation on composition and quality of table wines. Am. J. Enol. Vitic. 23, 152–158.
Renault, P., Miot-Sertier, C., Marullo, P., Henández-Orte, P., Lagarigue, L., Lonvaud-Funel, A., Bely, M., 2009. Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: potential applications in the wine industry. Int. J. Food Microbiol. 134, 201–210.
Ruiz, J., Belda, I., Beisert, B., Navascués, E., Marquina, D., Calderón, F., Rauhut, D., Santos, A., Benito, S., 2018. Analytical impact of *Metschnikowia pulcherrima* in the volatile profile of Verdejo white wines. Appl. Microbiol. Biotechnol. 102, 8501–8509.
Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J.J., Ballester, J., Vichi, S., Guérin-Schneider, R., Caixach, J., Alexandre, H., 2012. Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. Food Microbiol. 32, 243–253.
Santos, A., San Mauro, M., Bravo, E., Marquina, D., 2009. PMKT2, a new killer toxin from *Pichia membranifaciens*, and its promising biotechnological properties for control of the spoilage yeast *Brettanomyces bruxellensis*. Microbiol 155, 624–634.
Van der Walt, J.P., Van Kerken, A.E., 1958. The Wine Yeasts of the Cape. I. A Taxonomical Survey of the Yeasts Causing Turbidity in South African Table Wines. Wine Industry Research Group, Stellenbosch. South African Council for Scientific Industrial Research, Pretoria. https://link.springer.com/article/10.1007%2FBF02548451.
Van Sluyter, S.C., McRae, J.M., Falconer, R.J., Smith, P.A., Bacic, A., Waters, E.J., Marangon, M., 2015. Wine protein haze: mechanisms of formation and advances in prevention. J. Agric. Food Chem. 63 (16), 4020–4030.
Van Zyl, J.A., Du Plessis, L.d.W., 1961. The microbiology of South African winemaking. I. The yeasts occurring in vineyards, must and wines. S. Afr. J. Agric. Sci. 4, 393–403.