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Inoculation of *Torulaspora delbrueckii* as a bio-protection agent in winemaking

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ABSTRACT

In oenology, bio-protection consists in adding bacteria, yeasts or a mixture of microorganisms on grape must before fermentation in order to reduce the use of chemical compounds such as sulphites. More particularly, non-*Saccharomyces* yeasts are used as a total or partial alternative to sulphites. However, scientific data capable of proving the effectiveness of adding these yeasts on grape must is lacking. This study reports the analysis of antimicrobial and antioxidant effects of one non-*Saccharomyces* yeast, *Torulaspora delbrueckii*, inoculated at the beginning of the white winemaking process in two Burgundian wineries as an alternative to sulphiting. The implantation of the *T. delbrueckii* strain was successful in both wineries and had no impact on fermentation kinetics. Adding *T. delbrueckii* reduced biodiversity during the pre-fermentation stages compared to sulphited controls and it also effectively limited the development of spoilage microorganisms in the same way as the addition of sulphites. *T. delbrueckii* could protect must and wine from oxidation as demonstrated by the analysis of colour and phenolic compounds. This is the first evidence that early addition of *T. delbrueckii* during winemaking can be a microbiogical and chemical alternative to sulphites. However, its contribution seems to be matrix dependent.

1. Introduction

Sulphur dioxide (SO_2) is used as a preservative in oenology. This chemical additive has three main properties: it is antioxidant, antimicrobial and antioxidasic (Eschenbruch, 1986; Ribéreau-Gayon, Dubourbieu, Donèche, & Lonvaud, 2000). Its broad spectrum of action makes it indispensable for the winemaking process (Divol, du Toit, & Duckitt, 2012). Sulphites are generally used during prefermentation stages, after main fermentations and before bottling. However, the aims of modern oenology tend to decrease sulphites doses. One of the reason to reduce sulphite concentration in wines is the existence of sulphite sensitive consumers (Costanigro, Appleby, & Menke, 2014; Romano & Suzzi, 1993) particularly people who suffer from asthma (Vally & Thompson, 2001). These adverse effects encourage the reduction of sulphite addition by winemakers and the search for alternatives (Salaha, Kallithraka, Marmaras, Koussissi, & Tzourou, 2008). The maximum dose was reduced by 10 mg/L in 2009, in line with European regulations (Conventional wines: Regulation (EC) N° 606/2009 Annex 1B). The doses of total SO_2 decreased from 210 mg/L to 200 mg/L and 160 mg/L to 150 mg/L for conventional white and red dry wines, respectively. For organic wine, according to EU regulation (N° 203/2012) the total sulphites concentration should not exceed 100 mg/L and 150 mg/L for red and white wine, respectively. The new organic specifications, Demeter, Nature & Progrès (France), Swiss Bio and National organic program (USA), oblige very low sulphites doses, under 100 mg/L total SO_2. Chemical additives (ascorbic acid, sorbic acid, DMDC, etc.), heat treatment, the enzymatic method (lyzozyme) and the addition of inactivated yeasts enriched with glutathione and non-*Saccharomyces* yeasts are used to partially replace sulphites properties (Gao et al., 2002; Sonni, Chinnici, Natali, & Riponi, 2011). However, whatever the alternative used none of these products or methods have the same spectrum of action as sulphites. The use of non-*Saccharomyces* yeasts as a bio-preservative in must has been recently proposed by the wine industry as a possible alternative to sulphites.

Non-*Saccharomyces* (NS) yeasts have long been considered as spoilage flora, with the production of high levels of acetic acid and other

Abbreviations: DMDC, dimethyl dicarbonate; Td, *Torulaspora delbrueckii*; NS, non-*Saccharomyces*; SO_2, sulphur dioxide; EU, European Union; YPD, yeast peptone dextrose; TE, Tris EDTA; AF, alcoholic fermentation; MLF, malolactic fermentation; ADY, active dry yeast; DO, dissolved oxygen; UPLC, ultra-performance liquid chromatography; GRP, grape reaction product; PCA, principal component analysis; ANOVA, analysis of variance

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off-flavours (Fleet & Heard, 1993). However, their properties have been reviewed in recent decades (Gschaedler, 2017; Padilla, Gil, & Manzanares, 2016; Varela, 2016). Non-*Saccharomyces* yeasts are considered to be active in the first part of fermentation, when the ethanol concentration is not too high (Taillandier, Lai, Julien-Ortiz, & Brandam, 2014). Non-*Saccharomyces* species of interest participate in aroma enhancement through specific enzymatic activities such as glycosidases and β-lyase for terpene and thiol release, respectively (Esteve-Zarzoso, Manzanares, Ramon, & Querol, 1998; Gunata, Bayonove, Cordonnier, Arnaud, & Galzy, 1990; Zott, 2009). Some species such as *Metschnikowia pulcherrima* (Escott et al., 2018; Sadoudi et al., 2012; Sadoudi, Rousseaux, David, Alexandre, & Tourdot-Maréchal, 2017), *Pichia kluyveri* (Jolly, Augustyn, & Pretorius, 2017), *Kluyveromyces thermotolerans* (Benito, Calderón, & Benito, 2017; Del Fresno et al., 2017), *Candida zemplinina* (Zara et al., 2014), *Hanseniaspora vineae* (Medina et al., 2013) demonstrated oenological and aromatic contributions.

In the same way, *Torulaspora delbrueckii* has often been used in mixed alcoholic fermentation with *S. cerevisiae* in order to improve wine quality (Azzolini et al., 2012; Azzolini, Tosi, Lorenzini, Finato, & Zapparoli, 2015; Belda et al., 2015; Loira et al., 2014; Loira et al., 2015; Lu et al., 2017; Padilla et al., 2017; Puertas, Jiménez, Cantos-Villar, Cantoral, & Rodríguez, 2017; Ramírez et al., 2016; Velázquez, Zamora, Álvarez, Hernández, & Ramírez, 2015). Other biotechnological interest of this species has been largely described in the literature. It has been shown that *Torulaspora delbrueckii* is able to produce hydroxytyrosol known as bioactive compound (Álvarez-Fernández, Fernández-Cruz, Cantos-Villar, Troncoso, & García-Parrilla, 2018). *S. cerevisiae/T. delbrueckii* interaction can play a role by activating or inhibiting certain metabolic pathways (Tronchoni, Curiel, Morales, Torres-Pérez, & Gonzalez, 2017), especially in carbohydrate and nitrogen metabolism (Curiel, Morales, Gonzalez, & Tronchoni, 2017; García et al., 2017; Medina-Trujillo et al., 2017). Taking into account the properties of *T. delbrueckii*, wine companies are commercialising this species for oenological applications.

Inoculation on grapes or must has developed considerably in recent years (García, Esteve-Zarzoso, & Arroyo, 2016) in order to bio-protect the must by directly colonizing the environment and preventing the development of spoilage microorganisms. The industrial objective is to reduce the sulphites dose and to substitute its effect as much as possible. However, up to now no scientific data has supported or demonstrated the bio-protective effect of early yeast addition during the winemaking process.

This work aims at understanding the impact of a *T. delbrueckii* strain, used as a bio-protective agent instead of sulphites addition. We hypothesize that the addition of the yeast could compete with natural flora that prevents the development of spoilage microorganisms. Regarding potential protection against oxidation, this could be linked to the rapid consumption of oxygen by yeasts which prevents its utilisation by oxidative yeasts. In this study, we report for the first time an experiment performed at the industrial scale aimed at verifying the above hypothesis.

2. Materials and methods

2.1. Experiments in real winemaking conditions

Wine samples were produced with *Vitis vinifera* L. cv. Aligoté grapes from vineyards located in Burgundy wine-growing region in Northeast France. Burgundy wine region is characterized by a continental climate. The Aligoté crop is managed according to conventional viticulture practices in which chemical product are used for anti-*Oïdium* and anti-mildew treatment.

Grapes were harvested manually. Bio-protection by the addition of the strain *T. delbrueckii* BBMV 3FA5 (Primaflora VB®- AEB Group) was tested in two different wineries during the 2016 harvests. This strain was previously isolated in Burgundy and characterized in the laboratory (Sadoudi et al., 2012). *T. delbrueckii* or sulphites were added during juice extraction. The *T. delbrueckii* strain BBMV 3FA5 was added to 5×10^5 CFU/mL corresponding to the test modality (Td modality). For the control modality (S control modality), 30 mg/L SO_2 were added using a 5% sulphite solution. Exactly the same experiment was conducted in two different wineries using the same grape variety, the same winemaking process in order to have reproducible tests. In both wineries after pressing, musts were cold racked during 24 h before being inoculated (200 mg/L) with a commercial ADY *Saccharomyces cerevisiae*. Alcoholic fermentation was performed at 20 °C. At the end of alcoholic fermentation, the wines were inoculated with lactic acid bacteria to start malolactic fermentation. At the end of the winemaking process, the wines were bottled.

2.2. Experiments sampling

For each winery, samples (150 mL) were collected on must before the addition of the *T. delbrueckii* strain or sulphites (M1, M2), 3 h after the addition of yeast or SO_2, on racked must (Day 1, Day 2), at mid-alcoholic fermentation (MAF) (Day 5 or Day 7), and at the end of alcoholic fermentation (AF) (Day 10, Day 20) and malolactic fermentation (MLF).

2.3. Study of yeast diversity by Illumina MiSeq "paired-end" 2 × 250 bases

The analyses were carried out on must before the addition of the *T. delbrueckii* strain or sulphiting and after 3 h of vatting for both wineries. For each sample, 10 mL of must were collected and added in a polyamide column (2.5 g of MN Polyamid SC6 Macherey-Nagel + NaCl 1 M) in order to retain the maximum amount of polyphenols (Ye et al., 2011). The filtered suspension was centrifuged (3 min at 4 °C, at 16,000*g*) after which the pellet was suspended in 200 µL of yeast DNA (2% Triton X-100 (v/v), 1% SDS (w/v), 100 mM NaCl, 10 mM Tris, 1 mM EDTA pH 8) and 200 µL phenol/chloroform/isoamyl alcohol (50:48:2). The cell membrane was lysed by disruptor (Disruptor Genie®, scientific industries) for 3 min with 0.3 g of glass beads (diameter: 0.5 mm) and placed on ice for 2 min. 200 µL of TE (Tris EDTA) were added and the whole mixture was centrifuged for 10 min at 16,000*g* at 4 °C before collecting the aqueous phase. The DNA was precipitated with 2.5 volumes of 100% (v/v) ethanol and centrifuged at 16,000*g* at 4 °C for 10 min. The pellet was washed with 70% (v/v) ethanol, dried at 95 °C for 5 min and suspended in 40 µL of Milli-Q water (Grangeteau et al., 2016). The DNA concentrations were standardized (20 ng/µL) by measuring optical density at 260 nm and stored at −20 °C.

The samples were analysed using a metagenomic approach targeting 18 s rDNA. The libraries were compiled from extracts of pure genomic DNA according to the Metabiote protocol developed by Genoscreen (France) using the following PCR conditions. The primer set FR1 (59-AICCATTCAATCGGTAIT-39)/FF390 (59-CGATAACGAACGAG ACCT-39) were used. This primer set is located at the end of the SSU 18S rRNA gene, near the ITS1 region. The reaction conditions were as follows: an initial denaturation step of 94 °C for 2 min, then 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 7 min. PCR amplification with this primer set produces PCR fragments of ca. 350 bp.

This method allows a high-degree of multiplexing. The sequencing conditions consisted in loading on the "flow cell" of the final library at 4 pM. Demultiplexing was carried out using the CASAVA software (Illumina) after which a "merging" step provided the full-length sequences. The assembly parameter applied to 97% of nucleic identity allowed assembling the full-length 18S rDNA sequences at an average of 92.35%. Of high quality and in sufficient number, the full-length 18S rDNA sequences were then subjected to a clustering step. Operational Taxonomic Units (OTU) were created and compared to the SILVA database (Quast et al., 2013) by the Remote Desktop Software (RDP).

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