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Polysaccharides and Oligosaccharides Produced on Malvar Wines Elaborated with *Torulaspora delbrueckii* CLI 918 and *Saccharomyces cerevisiae* CLI 889 Native Yeasts from D.O. “Vinos de Madrid”

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ABSTRACT

Polysaccharides and oligosaccharides released into Malvar white wines elaborated through pure, mixed and sequential cultures with Torulaspora delbrueckii CLI 918 and Saccharomyces cerevisiae CLI 889 native yeasts from D.O. “Vinos de Madrid” were studied. Both fractions from different white wines were separated by high-resolution size-exclusion chromatography. Glycosyl composition and linkages of wine polysaccharides were determined by GC-EI-MS chromatography. Molar-mass distributions were determined by SEC-MALLS and intrinsic viscosity by differential viscometer. Yeast species and type of inoculation have a significant impact on wine carbohydrate composition and structure. Mannose residues from mannoproteins were significantly predominant in those cultures where T. delbrueckii was present in the fermentation process in comparison with pure culture of S. cerevisiae, whereas galactose residues from Polysaccharides Rich in Arabinose and Galactose presented higher values in pure culture of S. cerevisiae, indicating that S. cerevisiae released less mannoproteins than T. delbrueckii. Moreover, we reported structural differences between mannoproteins released by T. delbrueckii CLI 918 and those released by S. cerevisiae CLI 889. These findings help to provide important information about the polysaccharides and oligosaccharides released from cell wall of Malvar grapes and the carbohydrates released from each yeast species.

Keywords: Malvar white wines, native yeast, mannoproteins, polysaccharides, oligosaccharides
INTRODUCTION

The study of wine polysaccharides has acquired great interest in recent years owing to its role on a number of technological and sensorial properties in wine.

Numerous studies have clearly proven that these macromolecules have many positive oenological features such as protection against tartrate salt crystallization;\(^1\) prevention of protein haze formation in white wines;\(^2\) reduction of astringency;\(^3\)\(^,\)\(^4\) interaction with tannins;\(^5\) increased sweetness and increased body and mouth feel;\(^3\) formation of specific coordination complexes with Pb\(^{+2}\) ions.\(^6\) In sparkling wines, foam characteristics have been correlated with the type, the molecular weight and the glycosyl composition of polysaccharides.\(^7\)

Polysaccharides found in wine originate from grapes, yeasts and bacteria during the winemaking. Those originating from grape cell walls are Polysaccharides Rich in Arabinose and Galactose [PRAGs, which comprise arabinans, arabinogalactans, and arabinogalactan proteins],\(^8\) homogalacturonans\(^9\) and rhamnogalacturonans of type II (RG-II)\(^10\) whereas those released by microorganisms are mainly mannoproteins (MPs) produced by yeasts during the alcoholic fermentation or ageing on lees,\(^11\) and glucan-like structures by bacteria.\(^12\)

However, the oligosaccharides composition in wine have only recently been studied.\(^13\)\(^-\)\(^16\) These molecules are linked to defensive responses of plants.\(^17\)

Moreover, oligosaccharides can be found in important medicinal and food applications.\(^18\) According to their influence on the wine, it should be noted their ability to chelate cations which can be important for wines. The structure and the
amounts of oligosaccharides, as well as in the case of polysaccharides, depend on several factors, such as the grape origin\textsuperscript{19}, the grape cultivar\textsuperscript{16} and the winemaking process and can be modified by enzyme treatment.\textsuperscript{19} Therefore, it would appear to be necessary to go further with the identification, quantification and composition of oligosaccharides in different wines in order to better understand their influence on technological and organoleptic properties.

Recently, Giovani et al.\textsuperscript{20} have shown the high capacity of non-\textit{Saccharomyces} wine yeasts to release important polysaccharides into wine during alcoholic fermentation. The natural capacity of non-\textit{Saccharomyces} yeasts to release complex carbohydrates could be consider as selection criteria of these yeasts to wine elaboration.\textsuperscript{21} The role of non-\textit{Saccharomyces} yeasts in winemaking has been revised in recent years, the use of controlled mixed fermentations as a biotechnological tool has been promoted in order to enhance special and specific characteristics of a wine thus improve their complexity.\textsuperscript{21} This practice has also been reported as being able to increase some desirable metabolites, such as some acetate esters\textsuperscript{22} and glycerol.\textsuperscript{21} Moreover, some non-\textit{Saccharomyces} yeasts have been reported as being able to release more polysaccharides than \textit{S. cerevisiae} strains during alcoholic fermentation.\textsuperscript{20}

\textit{T. delbrueckii} was one of the first commercially available non-\textit{Saccharomyces} for the winemaking industry. During wine fermentation, \textit{T. delbrueckii} in pure cultures and mixed cultures with \textit{S. cerevisiae} has produced wines with higher sensory complexity and floral and fruity aromas because of its capacity of produce high concentration of higher alcohols, esters, terpenes and phenolic
aldehydes as well as other compounds like 2-phenylethanol and linalool.\textsuperscript{23} It has also been described its ability to produce wines with lower volatile acidity, acetaldehyde and acetoin.\textsuperscript{24} Depending on the strain, \textit{T. delbrueckii} can produce low/medium glycerol, succinic acid and polysaccharides.\textsuperscript{20,25,26} Moreover, \textit{Torulaspora} genera is reported good producer of enzymes such as $\beta$-glucosidases, pectinases, proteases and those related to xylan degradation but this capacity of enzymes secretion also depends on the yeast strain analyzed.\textsuperscript{27}

The Denomination of Origin (D.O.) “Vinos de Madrid”, created in 1990, is located in the centre of Spain (between 40° 16’ N and 40° 24’ N latitude) and covers an area of 8.390 ha. The climate of this agronomy zone is continental with temperatures ranging from $-8$ °C minimum in winter to 41 °C maximum in summer,\textsuperscript{28} and the annual rainfall means are between 460 and 660 mm. The most cultivated grape cultivars are Airén and Malvar (white), and Garnacha and Tempranillo (red) (all of them \textit{Vitis vinifera} L. cv.). Malvar is an autochthonous cultivar for this D.O., while Airén, Garnacha and Tempranillo have major extensions all over the Iberian Peninsula. Part of the economic development of this area is based on wine production with winemakers searching for the production of high quality wines with singular identity. The selection of native yeasts is an emerging tool which contributes to elaborate new styles of wines more competitive in the market.\textsuperscript{29}

The aim of this study was to determine the polysaccharides and oligosaccharides release into Malvar white wines elaborated with \textit{T. delbrueckii} CLI 918 and \textit{S. cerevisiae} CLI 889 native yeasts from D.O. “Vinos de Madrid” in pure, mixed
and sequential cultures made with both strains. To date, this is the first report of the polysaccharide and oligosaccharide composition in regional Malvar wines using autochthonous \textit{S. cerevisiae} and non-\textit{Saccharomyces} yeasts from this region.

**MATERIALS AND METHODS**

**Chemicals**

Analytical Reagent grade chemicals were used in the present study. Ammonium sulphate supplied by Panreac Quimica (Barcelona, Spain) and Enozym Altair pectolytic enzymes obtained from Agrovin (Ciudad Real, Spain), were used during must preparation. The following compounds were used by polysaccharides and oligosaccharides isolation and analysis in the studied wines. Sodium chloride, phosphorus pentoxide, hydrogen chloride, trifluoroacetic acid, ammonia, acetone, glacial acetic acid, ethyl acetate, acetic anhydride, perchloric acid 70%, 1-methylimidazole, chloroform, and n-hexane were obtained from Merck (Darmstadt, Germany). Methanol anhydrous, sodium borodeuteride and myo-inositol were purchased from Sigma-Aldrich (St Louis, MO, USA). Polyamide SC6 was supplied by Macherey-Nagel (Düren, Germany). Ammonium formiate was supplied by Acros Organics (Geel, Belgium). Tri-Sil HTP Reagent was obtained from Thermo Scientific (Waltham, MA, USA).

**Yeast strains**

The yeast strains used for the elaboration of the wines were the \textit{Saccharomyces} CLI 889 strain, selected as native strain from D.O. “Vinos de Madrid” and characterized in the IMIDRA’s laboratories based on some established and
desirable oenological criteria. This strain has been deposited in Spanish Type Culture Collection (CECT 13145). And *Torulaspora delbrueckii* CLI 918, selected according to its biotechnological potential. *T. delbrueckii* CLI 918 has been described earlier as a strain with potential interest for its contribution to the aromatic wine profile adding flowery and fruity notes and its use was considered interesting in mixed starter cultures with *S. cerevisiae*, and this strain has also shown good fermentative capacity under different stress conditions.

**Vinification procedure**

Grapes from Malvar cultivar (*Vitis vinifera* L. cv.) were hand-collected from IMIDRA’s experimental vineyard located in Madrid winegrowing region, Spain (40° 31’ N Longitude, 3° 17’ W Latitude, and 610 m Altitude) during the 2010 vintage at commercial maturity (21.5 °Brix, equivalent to about 205 g L$^{-1}$ of sugars). The grapes were gently destemmed and pressed and, the must was racked, homogenized and clarified by pectolytic enzymes (1 g/hL) at 4 °C. Clear must was supplemented with nitrogen by adding ammonium sulphate (NH$_4$)$_2$SO$_4$ up to a level of 250 mgN L$^{-1}$ before beginning the alcoholic fermentation.

Triplicate experiments were carried out in sterile flasks with 1 L of pasteurized (as indicated in García et al., 2017) Malvar must with constant agitation (150 rpm) in an 18 °C temperature controlled room. Fermentations were divided into pure, mixed and sequential cultures. Pure cultures were independently inoculated with $10^6$ cells mL$^{-1}$ CLI 889 *S. cerevisiae* strain (culture considered as control) and $10^6$ cells mL$^{-1}$ of CLI 918 *T. delbrueckii* strain. Mixed fermentation trials were simultaneously inoculated with $10^6$ cells mL$^{-1}$ of *T. delbrueckii* and $10^6$
cells mL⁻¹ of *S. cerevisiae* strain. Sequential fermentation trials, were inoculated with 10⁶ cells mL⁻¹ of the *T. delbrueckii* culture at first and the addition of the *S. cerevisiae* strain (10⁶ cells mL⁻¹) took place when the wine contained 5 % alcohol (v/v). The fermentation progress was monitored by automatic weight using the software OPCEEx3 (Resolvica Inc., Chanhassen, MN) every 24 h until the end of the fermentation (constant weight). After fermentation, yeast cells and wines were separated by centrifugation (9000 rpm, 10 min, 4 °C); the pellets were discarded and the wines were kept frozen until analysis.

Principal oenological parameters of final wines were measured by Fourier transform infrared spectroscopy in the laboratories of Liec Agroalimentaria S. L. (Manzanares, Spain), an accredited laboratory for physico-chemical analysis in wines to conform to UNE-EN ISO/IEC 17025:2005 rules.

**Isolation of polysaccharide and oligosaccharide fractions**

The polysaccharide and oligosaccharide fractions were isolated as previously described.⁴,⁷,¹³,¹⁵ Briefly, Malvar white wines (5 mL) were partially depigmented in polyamide CC6 columns, particle size 0.05–0.16 previously equilibrated with 1 M NaCl. Wine polysaccharides and oligosaccharides passed through the column and were eluted with two bed volumes of 1 M NaCl. The eluted fraction was concentrated in a centrifugal evaporator (EZ-2, Genevac, Ipswich, UK).

High-resolution size-exclusion chromatography (HRSEC) was performed by loading concentrated total wine carbohydrate on a system composed by a rheodyme sampling injector with a loop of 2 mL, an Intelligent pump 301 (FLOM, France), and a fraction collector Frac-920 (GE Healthcare Bio-Sciences,
Pittsburgh, USA). Elution was performed on a Superdex-30 HR column (60 x 1.6 cm, Pharmacia, Sweden) with a precolumn (0.6 x 4 cm) equilibrated at 1 mL min\(^{-1}\) with 30 mM ammonium formiate pH 5.6. The elution of polysaccharides and oligosaccharides was monitored with a RI 101 (Shodex Showa Denko, Japan) refractive index combined with Chromeleon software (Dionex, Sunnyvale, CA). The polysaccharide fraction was eluted between 41 and 55 min, while oligosaccharide fraction was collected between 56 and 93 min.\(^4\),\(^7\),\(^16\) The isolated fractions were freeze-dried, redissolved in water, and freeze-dried again four times to remove the ammonium salt.

**Glycosyl-Linkage determination**

The glycosyl-linkage compositions were determined by GC-MS of the partially methylated alditol acetates. One milligram of polysaccharides in 0.5 mL of dimethyl sulfoxide was methylated using methyl sulfinyl carbanion and methyl iodide.\(^{33}\) Methylated samples were hydrolyzed with 2 M of trifluoroacetic acid for 75 min at 120 °C. The released methylated monosaccharides were converted in their corresponding alditols by treatment with NaBD\(_4\) and then acetylated by adding ethyl acetate, acetic anhydride and perchloric acid.\(^{34}\) Partially methylated alditol acetates were analyzed by GC-EI-MS using a DB-1 capillary column (30 m x 0.25 mm i.d., 0.25 µm film), temperature programming 135 °C for 10 min and then 1.2 °C min\(^{-1}\) to 180 °C, with hydrogen as the carrier gas on a Shimadzu GCMS-QP2010SE gas chromatograph (Shimadzu, Kyoto, Japan).\(^{35}\) Myo-inositol was used as internal standard and, to verify the rate of methylation. Response factors of partially methylated alditol acetates are those described by Sweet et
The validity of the used methods and their repeatability were checked according to Vidal et al.\textsuperscript{35}

**Oligosaccharides analysis**

The neutral and acidic sugar compositions were determined after solvolysis with anhydrous MeOH containing 0.5 M HCl (80 °C, 16h), by GC of their per-O-trimethylsilylated methyl glycoside derivatives.\textsuperscript{37} The TMS derivatives were separated on two DB-1 capillary columns (30 m x 0.25 mm i.d., 0.25 µm film) (temperature programming 120–145 °C at 1.5 °C min\(^{-1}\), 145–180 °C at 0.9 °C min\(^{-1}\), and 180–230 °C at 50 °C min\(^{-1}\)), coupled to a single injector inlet through a two-holed ferrule, with hydrogen as the carrier gas on a Shimadzu GCMS-QP2010SE gas chromatograph. The outlet of the one column was directly connected to a FID at 250 °C, and the second column via a deactivated fused-silica column (0.25 m x 0.11 µm i.d.) was connected to a mass detector. Samples were injected in the pulse split mode with a split ratio of 20:1. The transfer line to the mass was set at 280 °C. Electron ionization mass spectra were obtained from m/z 50 to 400 every 0.2 s in the total ion-monitoring mode using an ion source temperature of 200 °C, a filament emission current of 60 µA, and an ionization voltage of 70 eV.

**Determination of molar mass of wine polysaccharides**

Molar mass distributions, molar weight and number-average mass (\(M_w\) and \(M_n\) in g/mol), polydispersity index (\(M_w/M_n\)), and intrinsic viscosity ([\(\eta\)] in mL g\(^{-1}\)) were determined at 25 °C by coupling size exclusion chromatography with a multiangle light scattering device (MALLS), a differential viscometer, and a
differential refractive index detector. SEC elution was performed on an OH-pack guard followed by two serial Shodex OH-pack KB-804 and KB805 columns (0.8 x 30 cm; Shodex Showa Denko, Japan) at a 1 mL min\(^{-1}\) flow rate in 0.1 M LiNO\(_3\) after filtration through a 0.1 µm filter unit. The MALLS photometer, a DAWN-HELEOS from Wyatt Technology Inc. (Wyatt Technology Corporation, Santa Barbara, CA, USA), was equipped with a GA–AS laser (λ = 658 nm). The differential viscometer detector (Viscostar II, Wyatt Technology Inc., USA) was equipped with a 4-capillary bridge design. The concentration of each eluted polysaccharide was determined using the differential refractive index detector (Optilab TrEX, Wyatt Technology Inc., USA). All collected data were analyzed using Astra V 6.0.6 software with the zimm plot (order 1) technique for molar-mass estimation and a differential refractive index increment of the polymer in the solvent used. A \(dn/dc\) classical value was employed for polysaccharides (0.146 mL g\(^{-1}\)).

Statistical treatment

One-factor ANOVA and Tukey HSD post-hoc tests were applied to establish the significance of differences between means (\(\alpha = 0.05\)). The data were analysed with SPSS Statistics 21.0 Software for Windows (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Fermentations

All fermentations finished in 27 days. Mixed culture of \(T. \text{delbrueckii}\) and \(S. \text{cerevisiae}\) (m-Td/Sc) presented a similar fermentation rate with regard to pure culture of \(S. \text{cerevisiae}\) (p-Sc, considered as control). In sequential culture of \(T. \text{delbrueckii}\) and \(S. \text{cerevisiae}\) (m-Td/Sc) presented a similar fermentation rate with regard to pure culture of \(S. \text{cerevisiae}\) (p-Sc, considered as control).
delbrueckii/S. cerevisiae (s-Td/Sc), the addition of S. cerevisiae (day 6 of fermentation; Sc in graphics) produced a significant increase in the fermentation rate (Figure 1). These co-cultures with T. delbrueckii and S. cerevisiae as well as the fermentation control p-Sc were characterized by an amount of residual sugar lower to 3 g L\(^{-1}\), which were therefore considered as dry wines. By contrast, T. delbrueckii in pure culture presented a lower fermentative capacity, did not consume the total quantity of sugars finishing with 33.70 g L\(^{-1}\) and 10.88 % of ethanol. It should also be highlighted the noticeably higher glycerol content found in pure and sequential of T. delbrueckii (7.14 and 5.28 g L\(^{-1}\), respectively) in comparison with mixed culture and the control (2.87 and 3.12 g L\(^{-1}\), respectively).

**Purification of polysaccharides and oligosaccharides fractions**

Malvar wines from pure, mixed and sequential cultures with T. delbrueckii and S. cerevisiae native yeasts were injected on Superdex 30-HR column in order to separate polysaccharides and oligosaccharides. The first two peaks eluted between 40 and 55 min corresponds to the polysaccharide fraction, it is composed of polysaccharides rich in arabinose and galactose (PRAGs), mannoproteins (MPs) and rhamnogalacturonans type II (RG-II).\(^6,39\) The polysaccharides rich in arabinose and galactose and rhamnogalacturonans come from the pecto-cellulosic cell walls of grape berries.\(^4,7,13,16,35\) The mannoproteins are released from the yeast during alcoholic fermentation.\(^35\) The fraction eluted in the range 56–93 min contained a complex mixture of small sugars which compose the oligosaccharides fraction of the different wines.\(^4,13,15,16,19\) Because
the winemaking process was the same, the profiles would indicate an important influence of yeast strain employed in the fermentation process and, the type of elaboration of the wines through pure, mixed or sequential culture could also be influential. Sequential culture of *T. delbrueckii* and *S. cerevisiae* presented the highest peaks during the purification process, which could suggest that the metabolism of both yeast strains have a cumulative effect (data not shown).

**Glycosyl-linkage composition of polysaccharides fraction**

The wine polysaccharides fraction was methylated, hydrolysed and analyzed for partially methylated alditol acetates by GC-MS. Their corresponding glycosyl-linkage composition is given in Table 1. Neutral sugars commonly present in wine polysaccharides can be found in Malvar wines (Table 1). The predominance of arabinose, galactose and mannose in the total colloids indicates that PRAGs from grape cell walls and mannoproteins from yeast cell walls were the major macromolecules in Malvar wines regardless of the type of inoculation used with *T. delbrueckii* and *S. cerevisiae*.

The major proportion of arabinosyl residues were present as terminal non-reducing position (2,3,5-tri-O-methyl ether) under furanose form, which may arise from both AGPs and arabinans. Arabinose linked →5 is characteristic of branched arabinans on the rhamnogalacturonan chain of the pectins (presence of 2- and 2,4-Rha). Values obtained for →5 arabinose linked are similar to commercial Champagne wines studies but noticeably lower than other red wines analyzed. It has been also detected arabinose linked at positions 2 and 3 with similar values regarding red wines, which origin are AGPs from grapes.
It also contained all methyl ether corresponding to the galactose linked at positions →3, →6, →3,6 and →3,4,6 linkages that are found in AGPs.\textsuperscript{8,10,35} The presence of 3,6-linked galactose indicates that type II arabinogalactans are side chains component of the RG-I in Malvar wines.\textsuperscript{8,10} The ratio of 3-linked galactose to 3,6-linked galactose residues (p-Sc, 1:3.1; p-Td, 1:2.9; m-Td/Sc, 1:2.7; s-Td/Sc, 1:3.0) indicated a highly-branched molecule similar to the AGP isolated from Carignan wine\textsuperscript{8} or Champagne wine.\textsuperscript{40} Vidal et al.\textsuperscript{3} reported that the addition of a mixture of AGPs and MPs have an influence on wine astringency and fullness. However, the physicochemical and sensorial properties related with PRAGs in wines need to be investigated.

Mannose residues were significantly predominant in those cultures where \textit{T. delbrueckii} CLI 918 strain has been used in the fermentation process. On the contrary, pure culture of \textit{S. cerevisiae} presented lower value of mannose residues (Table 1). Significant differences in molar percentage of mannose linked in →2, →3, →4 and →3,4 positions as well as in non-reducing terminal were observed, particularly for pure culture of \textit{T. delbrueckii} and sequential culture of \textit{T. delbrueckii}/\textit{S. cerevisiae} compared to pure culture of \textit{S. cerevisiae}. The glycosyl-linkage compositions of mannoproteins isolated from the Malvar wine inoculated by pure culture of \textit{S. cerevisiae} is in good agreement with accepted structure for glycosidic moiety of mannoproteins previously isolated from a Carignan red wine.\textsuperscript{35} The structure of mannoproteins in these fractions consisted of a long 6-linked backbone, highly substituted on position two with 2- and 3-linked mannose chains and attached to protein at asparagine units in the protein. These
structural characteristics are in agreement with the model proposed by Ballou for mannoproteins from Saccharomyces, and coincided with those typically found in yeast mannoproteins or with those mannoproteins released into the wine.

Nevertheless, it is important to highlight the structural differences of mannoproteins released by T. delbrueckii strain in comparison with those released by S. cerevisiae strain. In pure and sequential cultures with T. delbrueckii, the majority of mannoproteins were composed of short 2-linked mannose chains, attached to serine and threonine residues in the protein part (29.7% and 29.3% respectively) forming highly branched molecules. The ratio between the values (Table 1) exhibited for 2-linked mannose and terminal mannose in pure culture of T. delbrueckii presented a value of 4, therefore the chains of mannoproteins released by this non-Saccharomyces strain contain 4 residues of mannose linked in \( \rightarrow 2 \) for a terminal mannose. Mannose linked at 4-position has only been observed in pure culture of T. delbrueckii (Table 1), this 2,3,6-Man corresponds to the first mannose unit that it is 4-linked to the di-N-acetylchitobiose unit. In pure culture of S. cerevisiae (p-Sc) and in pure culture of T. delbrueckii (p-Td) the structure of mannoproteins released into the wine are different (Table 1).

Besides, it was also observed in co-cultures that the secreted mannoproteins will not be the same according to whether they are inoculated at the same time (m-Td/Sc: mixed culture of T. delbrueckii and S. cerevisiae) or in a sequential manner (s-Td/Sc: sequential culture of T. delbrueckii and S. cerevisiae). In mixed
culture (m-Td/Sc), mannoproteins have a glycosidic structure similar to that obtained with a pure culture of *S. cerevisiae* (p-Sc). And in sequential culture (s-Td/Sc), the structure of mannoproteins is similar to that of the pure culture of *T. delbrueckii* (p-Td) (Mannose linked in 2-position, Table 1). Furthermore, both pure cultures presented a different ability to release polysaccharides into the wine, showing higher ratio of polysaccharide release for *T. delbrueckii* than *S. cerevisiae*, these results are in agreement with the results obtained by Domizio et al.\(^{25}\) In the case of sequential culture, the polysaccharide release from *T. delbrueckii* CLI 918 could be explained as the result of actively growing cells of this non-*Saccharomyces* yeast strain before the inoculation of *S. cerevisiae* CLI 889. In the case of mixed culture of *T. delbrueckii* and *S. cerevisiae*, the fermentation process seems to be dominated by *S. cerevisiae* as the results were similar to the control (p-Sc). In reference to polysaccharides production in mixed cultures, Cominiti et al.\(^{43}\) observed a significant increase in the polysaccharides content only in the mixed fermentations of *T. delbrueckii/S. cerevisiae* at the inoculation ratios of 100:1 and 10,000:1. Other authors,\(^{44}\) using a spectroscopic approach to evaluate the polysaccharide/mannoprotein ratio has found differences depending on the yeast strain or species used.

Moreover, it is well known the relation of MPs with the retention of aromatic compounds and the increase of body and mouth feel in wines.\(^{3,45}\) After physico-chemical, aromatic and sensorial analysis of these wines, pure culture of *T. delbrueckii* and sequential culture of *T. delbrueckii* and *S. cerevisiae* contributed to increase the complexity and quality in the final wines. Specifically, sequential
culture was distinguished for their higher concentration of β-phenylethyl alcohol (rose aroma) and larger contents of esters such as 2-phenylethyl acetate, ethyl isovalerate and ethyl hexanoate. Sensorial analysis was carried out by tasting panel, tasters valued pure and sequential cultures of *T. delbrueckii* as the best ones due to their fruity and flowery aroma, higher aroma intensity and overall quality.32

**SEC-MALLS analysis of polysaccharides fractions from Malvar wines elaborated with *T. delbrueckii* and *S. cerevisiae* strains**

The polysaccharides relative index elution profiles show three principal populations (Figure 2). The concentration signal peaks are in the ranges 14.0–16.7 minutes for first population (P1), 16.7–18.8 minutes for second population (P2) and 18.8–20.5 minutes for third population (P3) (Figure 2, DRI signal). The molar mass of the eluting molecules decreased with increased elution volume in agreement with the normal size exclusion separation mechanism (Figure 2, $M_w$ signal). Different molecular parameters as molar mass, polydispersity index ($M_w/M_n$) and intrinsic viscosity ([η]) were measured in studied wines (Table 2). The molar mass appeared considerably higher for pure culture of *T. delbrueckii* in all three populations (P1: 682 000 g/mol; P2: 133 500 g/mol; P3: 33 300 g/mol) in comparison with other cultures. This result was in good agreement to González-Royo et al.,26 that described the presence of three populations of polysaccharides ($H M_w$: 144–1,000 kDa; $I M_w$: 40–144 kDa; $L M_w$: 5–40 kDa) in white wine fermented by sequential inoculation with *T. delbrueckii* and *S. cerevisiae*. The polydispersity index ($M_w/M_n$) was in general lower in third
populations (P3). All first populations (P1) showed higher values than values previously reported in sparkling red wines. These first populations (P1) (14.0–16.7 minutes) mainly corresponds to mannoproteins which molecular weights have been defined in the range from 50 000 to 560 000 g/mol. The intrinsic viscosity was notably lower for pure and sequential cultures of *T. delbrueckii* in the first population (P1), it may be related to the higher proportion of branched mannoproteins found in these two cultures. Previous publications established the influence of polysaccharides degree of branching on the intrinsic viscosity.46

The molar mass distribution analysis of the polysaccharides fractions from Malvar white wines elaborated with *T. delbrueckii* and *S. cerevisiae* yeasts is shown in Figure 3. Regarding these data, six delimited ranges among different wines can be observed (Molar mass range: range 1 = 2 500–20 000 g/mol; range 2 = 20 000–100 000 g/mol; range 3 = 100 000–250 000 g/mol; range 4 = 250 000–500 000 g/mol; range 5 = 500 000–1 000 000 g/mol; range 6 = 1 000 000–10 000 000 g/mol). Previous six ranges have been selected due to their correspondence with values obtained from different polysaccharides families by SEC analysis: RG-II monomer, $M_w = 5 000$ g/mol; RG-II dimer, $M_w = 10 000$ g/mol; MP$_{0c}$, $M_w = 58 000$ g/mol; AGP$_2$, $M_w = 165 000$ g/mol; MP$_{0a}$, $M_w = 350 000$ g/mol; MP$_3$, $M_w = 1 000 000$ g/mol.7,35 Also, AGP$_0$ with a molar mass around 145 000 g/mol was found in Carignan red wine.35

In pure culture of *T. delbrueckii* polysaccharides fractions, 0% of mass can be observed in range 1 which corresponds to polysaccharides like mannoproteins of small molar mass (and mainly RG-II in the case of red wines). However, values
in the range 1 in pure culture of *S. cerevisiae* (11%), mixed (8%) and sequential (10%) combinations presented values below the described in red wine.\(^7\) Obvious differences are also observable concerning the ranges 2 and 3. Pure culture of *T. delbrueckii* showed the highest values in the range 2 followed by mixed culture, pure culture of *S. cerevisiae* and sequential culture (Figure 3). On the contrary, this sequential culture presented higher percentage of mass in range 3, then pure culture of *T. delbrueckii*, mixed culture and pure culture of *S. cerevisiae*. In contrast, the percentage of mass in range 4, range 5 and range 6 were notably similar between four different types of cultures (Figure 3).

**Oligosaccharide composition**

There are no published studies about the glycosyl composition of oligosaccharides in Malvar varietal wines. In addition, this is the first time that the glycosyl composition and characteristic ratios of oligosaccharides in pure, mixed and sequential cultures with the native yeasts *T. delbrueckii* CLI 918 and *S. cerevisiae* CLI 889 has been analysed, showing the corresponding results in Table 3. The wines studied contain most of the sugars which take part in the composition of wine carbohydrates.\(^4,7,16,35\) These sugars are mainly rhamnose, arabinose, galactose, xylose, galacturonic acid and glucuronic acid coming from the pecto-cellulosic cell walls of grapes. The presence of xylose, glucuronic and 4-O-Me glucuronic acid residues indicated that traces of hemicellulose might be solubilized from grape berry cell walls.\(^13,47\) Other sugars such as mannose and glucose are released from yeast polysaccharides.
In our work, differences were observed in the predominance of several oligosaccharides between the types of cultures. However, the predominant oligosaccharides were glucose (20.0–40.9%), galacturonic acid (14.5–26.8%), xylose (11.1–15.3%), mannose (10.3–15.3%), arabinose (8.2–10.1%) and galactose (4.3–7.9%). 4-O-Me glucuronic acid (2.8–3.8%), rhamnose (1.8–2.9%) and xylitol (1.9–2.5%) were also found in all cultures but in lower quantities. The smallest quantities of oligosaccharides corresponded to glucuronic acid (1.1–1.8%) and fucose (1.4–1.6%) in agreement with results obtained in red and white still wines\(^4,13,14\) and sparkling wines.\(^7\) As shown in Table 3, glucose content released into the medium was higher (40.9%) in pure culture of \textit{S. cerevisiae} (culture considered as control) compared to the other samples. It could be possible that strains used in this work release a small part of their underlying layer composed of \(\beta\)-glucans, in addition to the external mannoprotein layer.\(^20\)

It could be highlighted the galacturonic acid content in pure culture of \textit{T. delbrueckii}. The high value of galacturonic acid has been reported in red wines before, and it has been explained by differences in the pectin composition and in the natural pectinase activities present in grape skins.\(^4,7,13,16\) In our case, it could be possible that the pectinase activity showed by \textit{T. delbrueckii} CLI 918 strain\(^28\) also influence this galacturonic acid value. It is well known that pectinase enzymes have a notable influence on technological and sensorial properties of wines.\(^48\)

Mannose residues were higher in wines elaborated with different combinations of \textit{T. delbrueckii} and \textit{S. cerevisiae}, in mixed and sequential inoculations (Table 3).
Mannose and glucose residues are mainly released from yeast cell walls, our findings regarding these two oligosaccharides are similar to those found by Quijada-Morín et al.\textsuperscript{4} and higher than obtained by others authors,\textsuperscript{7,13} probably due to differences in maturity stages between cultivars at time of the harvest or to different winemaking conditions.

The oligosaccharides coming from grape cell walls presented similar proportions in respect to those described in Chardonnay and Grignolino wines,\textsuperscript{14} with exception of galacturonic acid and mannose content that was considerably higher in Malvar wines. Oligosaccharides such as arabinose, rhamnose and galactose was larger in Carignan,\textsuperscript{13} Cabernet Sauvignon, Syrah and Monastrell\textsuperscript{16} red wines in comparison with Malvar white wines. It could be related to longer contact between grape skins and must during the elaboration of red wines than during the production of white ones.

In order to know the oligosaccharide sugar structures, several characteristic ratios have been calculated (Table 3): arabinose to galactose (Ara/Gal), rhamnose to galacturonic acid (Rha/Gal A), arabinose+galactose to rhamnose (Ara+Gal/Rha) and mannose to glucose (Man/Glc).

The ratio Ara/Gal is characteristic of the wine PRAGs.\textsuperscript{38,47} The value of this ratio has been previously described in red wines, close to 1.\textsuperscript{48} Other authors obtained ratios 2-fold higher in Carignan and Merlot red wines\textsuperscript{13} and in Tempranillo sparkling wines.\textsuperscript{7} In our work, this ratio showed significantly lower values in the case of sequential culture of \textit{T. delbrueckii} and \textit{S. cerevisiae} and pure culture of \textit{T. delbrueckii} compared to the control (Table 3). The higher ratio Ara/Gal in \textit{S. cerevisiae} CLI 918 and \textit{T. delbrueckii} CLI 918 native yeasts from D.O. “Vinos de Madrid”, Journal of Agricultural and Food Chemistry.
pure culture and mixed culture suggests a release of arabinose or oligosaccharides rich in arabinose coming from pectic framework. By contrast, it could be suggested that sequential inoculation produced a slight degradation of PRAG structures. This PRAG degradation has been described previously during post maceration and malolactic fermentation\textsuperscript{49} and during wine aging of lees by a partial dearabinosylation.\textsuperscript{47} Moreover, champagne wines presented a much lower ratio Ara/Gal (0.18) in comparison with red wines.\textsuperscript{40}

The Rha/Gal A ratio provides information on the relative richness of the wine oligosaccharides in homogalacturonans versus rhamnogalacturonans.\textsuperscript{50} There is a slight difference in the sequential culture. Although, these low ratios (between 0.1–0.2) for all the samples would suggest homogalacturonan predominance in oligosaccharides from Malvar wines. The results obtained were lower than those found in red still wines\textsuperscript{4,13,16,19} but higher than results obtained in red sparkling wines.\textsuperscript{7} This apparent discrepancy could be explained by the fact that grape variety could impact on wine oligosaccharide structure, as demonstrated by Apolinar-Valiente et al.\textsuperscript{16}

It is assumed that most of the Ara and Gal residues are associated with pectin hairy regions. Therefore, the (Ara+Gal) to rhamnose ratio was calculated to estimate the relative importance of the neutral side chains to the rhamnogalacturonan backbone. This ratio was considerably higher in pure culture of \textit{S. cerevisiae} in comparison with pure culture of \textit{T. delbrueckii}, mixed and sequential cultures (Table 3). It might be concluded that Malvar white wines elaborated from different \textit{T. delbrueckii}/\textit{S. cerevisiae} combinations contain more
structures from the hairy regions of pectins (rhamnogalacturonan-like structures
carrying neutral lateral chains) as a result of breakdown of grape cell wall berries
by pectinases. This degradation could be favoured by pectinase activity presented
by *T. delbrueckii* CLI 918 strain. Different values for this ratio have been found
in other grape varieties, 2.8 and 3.1 in Carignan and Merlot oligosaccharides;\(^\text{13}\)
2.8 for Syrah oligosaccharides, 4.7 in Cabernet Sauvignon grape variety and 5.1
in Monastrell variety.\(^\text{16}\) Also, Martínez-Lapuente et al.\(^\text{7}\) showed the change of
(Ara+Gal)/Rha ratio in Tempranillo sparkling wines during the aging.
The Man/Glc ratio has been related with the effectiveness of mannoproteins for
protein stabilization in white wines, being more effective when this proportion is
higher.\(^\text{45}\) In our case, glucose was the largely major residue sugar (20.0–40.9%)
whereas mannose represented smaller proportions (10.3–15.3%). Glucose is the
prevalent sugar in grape berries\(^\text{9}\) since it is the main component of cellulose and
hemicellulosic xyloglucans. Furthermore, the presence of glucose in wines may
also be related to microbial cell walls (*Botryotinia fuckeliana*, *Oenococcus oeni*)
or condensed anthocyanins.\(^\text{39}\) In this work, grapes were harvested in good
sanitary conditions, Malvar white grapes did not contain anthocyanins, and
malolactic fermentation was not conducted. Therefore, the glucose content in
these Malvar wines would come from yeast glucans released during the
fermentation. Because the different chemical composition indicated by the
Man/Glc ratio for oligosaccharides could determine a different functional effect
on the wine, further analyses will be necessary to determine a possible
correlation on the wine attributes derived from the different mannoprotein
released through pure, mixed or sequential culture of *T. delbrueckii* CLI 918 and *S. cerevisiae* CLI 889.

Taking into account that the grape variety, maturity stage and grape processing were equal, the results of this study highlight that yeast strain and type of inoculation have a significant impact on wine carbohydrate composition and structure. Regarding the composition of polysaccharides fraction, mannose residues from MPs were significantly predominant in those cultures where *T. delbrueckii* was present in the fermentation process, whereas galactose residues from PRAGs presented higher values when pure culture of *S. cerevisiae* was employed, indicating that *S. cerevisiae* released less mannoproteins than *T. delbrueckii*. Concerning the molecular parameters, the molar mass appeared considerably higher for pure culture of *T. delbrueckii* in comparison with other cultures, appearing also variations with regard to the intrinsic viscosity depending on the population observed by RI technique. Moreover, the molar mass distribution of the polysaccharides fractions from Malvar white wines also showed obvious changes relied on the yeast strain and type of inoculation used.

As regards the polysaccharide fractions in pure culture of *T. delbrueckii*, 0% of molar mass can be observed in the range between 2 500 and 20 000 g/mol whereas the control (p-Sc) and the co-cultures presented values between 8.4 and 10.9 % for this range. Clear differences were also observable in the range between 20 000 and 100 000 g/mol and the range between 100 000 and 250 000 g/mol. The analysis of Malvar wines has also revealed that the oligosaccharide
composition and structure could be significantly influenced by the types of cultures.

In summary, our results provide relevant information about the polysaccharides and oligosaccharides released from cell wall of Malvar grapes and the carbohydrates released from cell wall of different yeast strains through pure, mixed or sequential cultures of *T. delbrueckii* CLI 918 and *S. cerevisiae* CLI 889. These findings, about the use of different types of cultures, aim to improve the quality of wines from D.O. “Vinos de Madrid”, highlighting especially sequential cultures to produce larger amounts of mannoproteins enhancing the complexity and quality of Malvar wines. Analysis should be carried out to deepen our knowledge concerning the capacity of *Saccharomyces* and non- *Saccharomyces* yeast strains to release mannoproteins, which could be considered a selection criterion for wine elaboration because of their reported contribution to wine quality.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

PRAGs, polysaccharides rich in arabinose and galactose; AGs, type II arabinogalactans; AGPs, type II arabinogalactan-proteins; RG-I, rhamnogalacturonans type I; RG-II, rhamnogalacturonans type II; MPs, mannoproteins; TMS, per-O-trimethylsilylated methyl glycoside; HRSEC, high-resolution size-exclusion chromatography; GC-EI-MS, gas chromatography electron ionization mass spectrometry; SEC-MALLS, size exclusion chromatography-multiangle laser light scattering.
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polysaccharide composition in grape (*Vitis vinifera* L.) and apple (*Malus
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Table 1. Glycosyl-Linkage Composition (mole percentage) of polysaccharides fractions isolated from Malvar wines elaborated with different types of inoculation of the yeast strains *T. delbrueckii* CLI 918 and *S. cerevisiae* CLI 889. Values are the mean ± SD of fermentations.

<table>
<thead>
<tr>
<th>Methyl ether</th>
<th>Linkage</th>
<th>p-Sc&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p-Td&lt;sup&gt;b&lt;/sup&gt;</th>
<th>m-Td/Sc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>s-Td/Sc&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>234 Rhamnose</td>
<td>Terminal</td>
<td>2.9 ± 0.8</td>
<td>1.3 ± 0.1*</td>
<td>2.2 ± 0.4</td>
<td>1.9 ± 0.2*</td>
</tr>
<tr>
<td>34 Rhamnose</td>
<td>2-Linked</td>
<td>1.8 ± 0.5</td>
<td>0.8 ± 0.2*</td>
<td>1.4 ± 0.4</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>3 Rhamnose</td>
<td>2,4-Linked</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.0*</td>
</tr>
<tr>
<td>Total rhamnose&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>5.1 ± 1.4</td>
<td>2.4 ± 0.2*</td>
<td>3.8 ± 0.2</td>
<td>3.1 ± 0.8*</td>
</tr>
<tr>
<td>235 Arabinose</td>
<td>Terminal furanose</td>
<td>13.3 ± 3.3</td>
<td>11.0 ± 0.7</td>
<td>15.9 ± 3.5</td>
<td>12.2 ± 2.2</td>
</tr>
<tr>
<td>25 Arabinose</td>
<td>3-Linked</td>
<td>1.6 ± 0.4</td>
<td>0.6 ± 0.1*</td>
<td>0.2 ± 0.0*</td>
<td>0.6 ± 0.2*</td>
</tr>
<tr>
<td>35 Arabinose</td>
<td>2-Linked</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>23 Arabinose</td>
<td>5-Linked</td>
<td>0.4 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Total arabinose&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>15.7 ± 3.9</td>
<td>12.7 ± 0.9</td>
<td>17.0 ± 3.6</td>
<td>13.9 ± 2.8</td>
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<tr>
<td>2346 Galactose</td>
<td>Terminal</td>
<td>3.3 ± 0.4</td>
<td>2.5 ± 0.6</td>
<td>3.0 ± 0.8</td>
<td>2.8 ± 0.6</td>
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<tr>
<td>234 Galactose</td>
<td>6-Linked</td>
<td>8.1 ± 0.6</td>
<td>8.8 ± 0.1</td>
<td>8.1 ± 0.1</td>
<td>7.1 ± 0.8*</td>
</tr>
<tr>
<td>246 Galactose</td>
<td>3-Linked</td>
<td>5.6 ± 0.3</td>
<td>5.6 ± 0.1</td>
<td>5.9 ± 1.2</td>
<td>4.3 ± 0.9</td>
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<tr>
<td>24 Galactose</td>
<td>3,6-Linked</td>
<td>17.7 ± 1.3</td>
<td>16.3 ± 0.8</td>
<td>16.4 ± 2.1</td>
<td>13.1 ± 0.8*</td>
</tr>
<tr>
<td>2 Galactose</td>
<td>3,4,6-Linked</td>
<td>4.2 ± 0.6</td>
<td>3.5 ± 0.4</td>
<td>3.2 ± 0.4</td>
<td>2.7 ± 0.7*</td>
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<tr>
<td>Total galactose&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>38.9 ± 2.9</td>
<td>36.7 ± 1.8</td>
<td>36.6 ± 4.0</td>
<td>30.0 ± 2.4*</td>
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<tr>
<td>2346 Glucose</td>
<td>Terminal</td>
<td>1.4 ± 0.5</td>
<td>1.0 ± 0.1</td>
<td>0.4 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>234 Glucose</td>
<td>6-Linked</td>
<td>1.2 ± 0.9</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>1.5 ± 0.8</td>
</tr>
<tr>
<td>Total glucose&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>2.6 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>0.8 ± 0.6*</td>
<td>1.8 ± 0.9</td>
</tr>
<tr>
<td>2346 Mannose</td>
<td>Terminal</td>
<td>11.8 ± 0.1</td>
<td>7.0 ± 0.1*</td>
<td>12.3 ± 0.8</td>
<td>11.4 ± 0.4</td>
</tr>
<tr>
<td>346 Mannose</td>
<td>2-Linked</td>
<td>7.2 ± 0.8</td>
<td>29.7 ± 2.4*</td>
<td>12.3 ± 3.9*</td>
<td>29.3 ± 2.3*</td>
</tr>
<tr>
<td>246 Mannose</td>
<td>3-Linked</td>
<td>8.3 ± 0.4</td>
<td>0.0 ± 0.0*</td>
<td>7.8 ± 0.2</td>
<td>3.2 ± 0.7*</td>
</tr>
<tr>
<td>236 Mannose</td>
<td>4-Linked</td>
<td>0.0 ± 0.0</td>
<td>2.6 ± 0.7*</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>34 Mannose</td>
<td>2,6-Linked</td>
<td>8.8 ± 1.6</td>
<td>6.1 ± 0.5</td>
<td>8.1 ± 2.8</td>
<td>6.5 ± 1.7</td>
</tr>
<tr>
<td>26 Mannose</td>
<td>3,4-Linked</td>
<td>2.0 ± 0.5</td>
<td>2.0 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>1.3 ± 0.1*</td>
</tr>
<tr>
<td>Total mannose&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>38.1 ± 3.3</td>
<td>47.4 ± 2.1*</td>
<td>42.2 ± 7.6</td>
<td>51.7 ± 0.3*</td>
</tr>
</tbody>
</table>

a Relative molar percent of each parent sugar family (sum of ethers from one sugar type) within total sugars.

b p-Sc: pure culture of *S. cerevisiae*; p-Td: pure culture of *T. delbrueckii*; m-Td/Sc: mixed culture of *T. delbrueckii* and *S. cerevisiae*; s-Td/Sc: sequential culture of *T. delbrueckii* and *S. cerevisiae*.

* Means statistically different from the control (p-Sc), p<0.05
Table 2. Parameters\(^a\) obtained for the polysaccharides isolated from Malvar white wines elaborated with \(T.\ delbrueckii\) and \(S.\ cerevisiae\) strains using different type of inoculation.

<table>
<thead>
<tr>
<th>Wine sample(^b)</th>
<th>Peak(^c)</th>
<th>(M_n) (g/mol)</th>
<th>(M_n) (g/mol)</th>
<th>(M_w/M_n)</th>
<th>Intrinsic Viscosity (mL/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Sc</td>
<td>1</td>
<td>539 000</td>
<td>422 200</td>
<td>1.31</td>
<td>43.59</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>118 850</td>
<td>97 390</td>
<td>1.21</td>
<td>17.34</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>18 975</td>
<td>16 595</td>
<td>1.14</td>
<td>9.36</td>
</tr>
<tr>
<td>p-Td</td>
<td>1</td>
<td>682 000</td>
<td>522 100</td>
<td>1.31</td>
<td>33.32</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>133 500</td>
<td>108 200</td>
<td>1.23</td>
<td>16.30</td>
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<tr>
<td></td>
<td>3</td>
<td>33 300</td>
<td>30 300</td>
<td>1.10</td>
<td>7.03</td>
</tr>
<tr>
<td>m-Td/Sc</td>
<td>1</td>
<td>679 350</td>
<td>453 600</td>
<td>1.50</td>
<td>51.99</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>124 200</td>
<td>102 600</td>
<td>1.22</td>
<td>16.81</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>26 490</td>
<td>22 990</td>
<td>1.15</td>
<td>8.32</td>
</tr>
<tr>
<td>s-Td/Sc</td>
<td>1</td>
<td>491 000</td>
<td>387 600</td>
<td>1.27</td>
<td>39.07</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>129 100</td>
<td>115 550</td>
<td>1.12</td>
<td>17.99</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25 620</td>
<td>22 575</td>
<td>1.13</td>
<td>10.76</td>
</tr>
</tbody>
</table>

\(^a\) Molar-mass distribution, \(M_w\) (Molar weight), \(M_n\) (Number-average mass), \(M_w/M_n\) (Polydispersity index), and Intrinsic viscosity ([\(\eta\)]).

\(^b\) Abbreviations related with the type of culture employed and the yeast strains are explained in Table 1.

\(^c\) Peak 1: ranges 14.0 – 16.7 min (first population, P1); peak 2: ranges 16.7 – 18.8 min (second population, P2); peak 3: ranges 18.8 – 20.5 min (third population, P3).
Table 3. Glycosyl composition (mole percentage) and characteristics ratios of oligosaccharides isolated from Malvar wines elaborated with *T. delbrueckii* CLI 918 strain and *S. cerevisiae* CLI 889 strain using different inoculation strategies. Values are the mean ± SD of fermentations.

<table>
<thead>
<tr>
<th></th>
<th>p-Sc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p-Td&lt;sup&gt;b&lt;/sup&gt;</th>
<th>m-Td/Sc&lt;sup&gt;b&lt;/sup&gt;</th>
<th>s-Td/Sc&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rha&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8 ± 0.9</td>
<td>2.9 ± 0.3*</td>
<td>2.7 ± 0.1*</td>
<td>2.7 ± 0.1*</td>
</tr>
<tr>
<td>Fuc</td>
<td>1.0 ± 0.3</td>
<td>1.4 ± 0.1*</td>
<td>1.4 ± 0.1*</td>
<td>1.6 ± 0.1*</td>
</tr>
<tr>
<td>Ara</td>
<td>8.2 ± 1.0</td>
<td>10.0 ± 2.2</td>
<td>10.1 ± 0.2</td>
<td>9.6 ± 0.4</td>
</tr>
<tr>
<td>Gal</td>
<td>4.3 ± 1.4</td>
<td>7.9 ± 0.4*</td>
<td>6.6 ± 0.4*</td>
<td>7.9 ± 0.3*</td>
</tr>
<tr>
<td>Glc</td>
<td>40.9 ± 1.1</td>
<td>20.0 ± 1.4*</td>
<td>21.6 ± 2.9*</td>
<td>26.4 ± 0.9*</td>
</tr>
<tr>
<td>Man</td>
<td>10.3 ± 0.3</td>
<td>12.4 ± 2.2</td>
<td>15.1 ± 1.6*</td>
<td>15.3 ± 0.8*</td>
</tr>
<tr>
<td>Xyl</td>
<td>11.1 ± 0.8</td>
<td>12.2 ± 2.8</td>
<td>15.3 ± 0.1*</td>
<td>15.1 ± 0.3*</td>
</tr>
<tr>
<td>Gal A</td>
<td>16.7 ± 1.8</td>
<td>26.8 ± 3.0*</td>
<td>19.9 ± 0.7</td>
<td>14.5 ± 1.1</td>
</tr>
<tr>
<td>Glc A</td>
<td>1.1 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.8</td>
</tr>
<tr>
<td>4-O-MeGlc A</td>
<td>2.9 ± 0.2</td>
<td>3.4 ± 0.1*</td>
<td>3.8 ± 0.0*</td>
<td>2.8 ± 0.0</td>
</tr>
<tr>
<td>Xylitol</td>
<td>1.9 ± 0.4</td>
<td>1.9 ± 0.3</td>
<td>2.1 ± 0.4</td>
<td>2.5 ± 0.2*</td>
</tr>
</tbody>
</table>

| Ratio    | Ara/Gal          | 2.0 ± 0.4        | 1.3 ± 0.5*          | 1.6 ± 0.1           | 1.2 ± 0.1*          |
| Rha/Gal A | 0.1 ± 0.0        | 0.1 ± 0.0        | 0.1 ± 0.0           | 0.2 ± 0.0*          |
| (Ara+Gal)/Rha | 7.9 ± 2.8     | 6.2 ± 0.4        | 6.2 ± 0.4           | 6.5 ± 0.4           |
| Man/Glc  | 0.2 ± 0.0        | 0.6 ± 0.1*       | 0.7 ± 0.2*          | 0.6 ± 0.1*          |

<sup>a</sup> Rha, Rhamnose; Fuc, Fucose; Ara, Arabinose; Gal, Galactose; Glc, Glucose; Man, Mannose; Xyl, Xylose; Gal A, Galacturonic acid; Glc A, Glucuronic acid; 4-O-MeGlc A, 4-O-methyl Glucuronic acid.

<sup>b</sup> Abbreviations related with the type of culture employed and the yeast strains are explained in Table 1.

<sup>*</sup> Means statistically different from the control (p-Sc), p<0.05
FIGURE CAPTIONS

Figure 1. Fermentation kinetics of pure (p), mixed (m) and sequential (s) cultures in Malvar must with *T. delbrueckii* (Td) and *S. cerevisiae* (Sc) yeast strains. Values are the means from triplicate fermentations.

Figure 2. SEC-MALLS chromatograms and weight-average molar mass distributions of the polysaccharide fraction in pure (p), mixed (m) and sequential (s) cultures made with *T. delbrueckii* (Td) and *S. cerevisiae* (Sc) strains. Molar weight distribution ($M_w$; g/mol; continuous line) and refractive index (DRI; relative scale; dashed line).

a Peak 1: ranges 14.0 – 16.7 min (first population, P1); peak 2: ranges 16.7 – 18.8 min (second population, P2); peak 3: ranges 18.8 – 20.5 min (third population, P3).

Figure 3. Distribution analysis determined by light scattering ($dn/dc = 0.146$ mL/g) of polysaccharides fractions isolated from Malvar white wines elaborated with *T. delbrueckii* and *S. cerevisiae* yeast strains with different type of inoculation.

a Abbreviations related with the type of culture employed and the yeast strains are explained in Table 1.
Figure 1

![Graph showing CO₂ released (g L⁻¹) over time (days) for different samples labeled as p-Sc, p-Td, m-Td/Sc, and s-Td/Sc. The graph includes a time axis from 0 to 27 days and a CO₂ release axis from 0 to 140 g L⁻¹.](image-url)
Figure 2
Figure 3
TOC graphic