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ORIGINAL PAPER

Effect of sequential inoculation (*Torulaspora delbrueckii*/*Saccharomyces cerevisiae*) in the first fermentation on the foaming properties of sparkling wine

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Abstract In a previous study, we reported that sequential inoculation of *Torulaspora delbrueckii* (strain TD291) and a specific strain of *Saccharomyces cerevisiae* during the first fermentation increased the protein concentration and improved the foaming properties of a base wine. Since effervescence and foam of sparkling wines are key quality factors, the interest of this practice for sparkling wine industry is obvious. In this paper, we study whether the foaming properties of the sparkling wines produced from the base wines obtained by sequential inoculation with these strains of *T. delbrueckii* and *S. cerevisiae* remain better than those of their controls produced from base wines fermented only with *S. cerevisiae*. The obtained results confirmed that sequential inoculation in the production of the base wine originated sparkling wines with significantly higher maximum heights of foam than conventional inoculation, probably because autolysis of the *T. delbrueckii* (strain TD291) cells in the base wine released higher amounts of proteins, especially of the low molecular weight fraction.

Keywords *Torulaspora delbrueckii* · *Saccharomyces cerevisiae* · Sequential inoculation · Sparkling wine · Foam

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Introduction

The quality of sparkling wines depends on several factors, including effervescence and foam [1]. One of the main concerns of the sparkling wine industry is therefore to find new ways of improving foamability and foam persistence. The foaming properties of sparkling wines depend largely on their chemical composition [2], which is closely related to grape variety and maturity and the winemaking conditions [3]. It has been reported that foam stability is favored by the presence of surfactant agents such as proteins, mannoproteins and polysaccharides, whose surface properties stabilize the interface of the bubbles [4]. In fact, it has been reported that all winemaking treatments that decrease the protein concentration dramatically affect the wine's foaming properties [5, 6]. The sparkling wine industry is therefore usually extremely careful with every factor that affects the protein levels of musts, base wines and sparkling wines, such as the maturity level [7], the pressing process [3], discoloration with charcoal [8], bentonite fining [9, 10] and the use of certain riddling agents [11]. Several strategies for improving foam properties have also been proposed. These include supplementation with inactive dried yeasts [12] and the selection of different strains of *Saccharomyces cerevisiae* with higher autolytic capacities [13].

It is well know that the development of some non-*Saccharomyces* yeasts such as *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Candida zemplinina*, *Hanseniaspora spp* and *Pichia kluyveri* during alcoholic fermentation can improve the quality and complexity of wine [14, 15]. Specifically, positive effects have been reported on aroma, glycerol, polysaccharides, mannoproteins and volatile acidity either by using mixed cultures or through the sequential inoculation of different species of non-*Saccharomyces* and *Saccharomyces* [16–19]. Some strains of these

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non-Saccharomyces yeasts are available in the market in the form of active dry yeast, and the International Organization of Vine and Wine (OIV) is currently updating its information on non-*Saccharomyces* yeasts.

Torulaspora delbrueckii/ is one of the most promising non-*Saccharomyces* yeasts because it increases glycerol concentration, reduces the content of acetic acid and acetaldehyde and enhances total acidity of wines [14, 20]. It has also been reported that *T. delbrueckii* increases the presence of certain volatile compounds [21] because of its higher β-glucosidase activity. Some strains of *T. delbrueckii* also appear to have a greater polysaccharide production capacity than *S. cerevisiae* [22, 23]. More recently, our research group has proposed using *T. delbrueckii* to improve the foaming properties of sparkling wines [24]. A sequential inoculation of *T. delbrueckii* (strain TD291) and a specific strain of *S. cerevisiae* during the first fermentation increased the protein concentration and both the maximal (HM) and stable height (HS) of the foam of the base wine by the Mosalux method. However, it has not yet been verified whether this effect remains in sparkling wine after the second fermentation. In this paper, we study whether sparkling wines produced from the base wines obtained by sequential inoculation with *T. delbrueckii* and *S. cerevisiae* present better foaming properties than their controls produced from base wines fermented only with *S. cerevisiae.*

Materials and methods

Chemicals

All products were of high purity and suitable for high-performance liquid chromatography (HPLC). Ammonium formate and ammonium acetate with grade purity ≥99.0 % were purchased from Sigma–Aldrich (Madrid, Spain). Absolute ethanol was supplied by VWR (Fontenay-sous-Bois, France). Hydrochloric acid and sodium hydroxide were provided by Panreac (Barcelona, Spain). Pure water was obtained from a Milli-Q purification system (EMD Millipore, Billerica, MA, USA).

Base wines

White monovarietal base wines were produced in the experimental winery of the Enology Faculty of the Rovira i Virgili University in Constantí (Tarragona, Spain) with Macabeo grapes from vineyards belonging to Juve & Camps SL in Espiells [AOC Cava; 41°27′ 1.8972″ (N) and 1°49′ 6.6216″ (E)] during the 2013 vintage and as described in a previous study [24]. The grapes were picked manually, crushed and pressed to obtain a yield of 0.6 L/kg of grapes. The must was sulphited immediately (30 mg/L of potassium disulphite) and filtered with a rotary vacuum filter. The grape juice was then poured into six stainless steel tanks each with a capacity of 100 L. Three tanks were conventionally inoculated with 250 mg/L of a commercial *S. cerevisiae* yeast strain (QA23®, Lallemand Inc., Montreal, Canada), and the other three tanks were initially inoculated with 250 mg/L of commercial *T. delbruekii* (strain TD291, Biodiva™, Lallemand Inc., Montreal, QC, Canada). Twenty-four hours later, when the density had fallen to around ten units, the tanks were reinoculated with 250 mg/L of the control *S. cerevisiae* yeast strain (QA23®, Lallemand Inc., Montreal, QC, Canada). As shown in a previous study [24], the imposition of the commercial strains of *T. delbruekii* and *S. cerevisiae* was verified.

All microvinifications were performed at 18 ± 1 °C. Once the alcoholic fermentations had finished, the wines were racked and sulphited (40 mg/L of potassium disulphite). All wines were maintained in airtight vessels at 4 °C. Samples of the base wines were analyzed three months later.

Sparkling winemaking

Sparkling wine (Cava) production was carried out by the traditional method with both base wines six months after the end of alcoholic fermentation. Both base wines were supplemented with 22 g L^{-1} of sucrose, 30 mg L^{-1} of bentonite as the riddling agent (Adjuvant 83; Station Oenotechnique du Champagne, Epernay, France) and 2 × 10^6 cells ml^{-1} of a preadapted yeast culture (EC1118, Lallemand Inc., Montreal, QC, Canada). Twelve bottles of each base wine were prepared. The wines were then bottled and crown corked. Nine months later, all the sparkling wines were disgorged, analyzed and tasted.

Preparation of samples for analysis

The sparkling wines were centrifuged at 12,000 × g at 4 °C for 15 min (Sorvall RC-5C, Heraeus, Hanau, Germany). The supernatant was used directly to measure the foaming properties and for chemical analysis (pH and ethanol content). For other analyses and to isolate the macromolecular fraction, the samples were previously degassed by magnetic stirring in an Erlenmeyer flask for 15 min.

Standard wine analysis

The analytical methods recommended by the OIV were used to determine titratable acidity and volatile acidity [25]. Ethanol contents (% v/v) were measured using an ebulliometer (GAB systematic analytical, Barcelona, Spain), and pH was measured using a pH meter Basic-20 (CRISON, Barcelona, Spain). Glycerol and sugars (D-glucose and

D-fructose) were measured using enzymatic kits (R-Biopharm AG., Darmstadt, Germany).

Supplementation of a still wine with macromolecular fraction from different sparkling wines

Aliquots of 300 mL of both previously degassed sparkling wines were dialyzed in tubes with a molecular weight cutoff of 3.5 kDa (Membrane Filtration Products Inc., San Antonio, TX, USA). The dialyzed samples were lyophilized and preserved at −20 °C. All the macromolecules contained in 300 mL of each of the two sparkling wines were dissolved in 450 mL of a still white wine in order to enrich it in the colloids. The original wine and the enriched wines were used to analyze the foaming properties, the polysaccharides and the proteins.

Measurement of the foaming properties

The samples were tempered at 18 °C for 24 h before analysis. Foamability and foam stability were measured using the Mosalux method [26]. A glass cylinder placed on a glass frit was filled with 100 mL of the sample. Carbon dioxide was then injected into the glass cylinder through the glass frit, with a constant gas flow of 115 mL/min under a constant pressure of 2 bars.

Two parameters were measured using a Mosalux apparatus (Station Oenotechnique de Champagne, Cormontreuil, France): HM, which is the maximum height reached by the foam after CO_2 injection through the glass frit; and HS, which is the stable height during CO_2 injection. HM represents foamability (the wine's ability to foam), and HS represents foam stability (the persistence of the foam collar, or the wine's ability to have a stable foam). Both of these parameters are expressed in mm. The Mosalux parameters were determined three times for each bottle of sparkling wine, which was analyzed in triplicate (3 bottles × 3 replicates per bottle).

Preparation of sample proteins and determination by HRSEC-DAD

Aliquots of sparkling wines (20 mL) were dialyzed in tubes with a molecular weight cutoff of 3.5 kDa (Membrane Filtration Products Inc., San Antonio, TX, USA). The dialyzed samples were lyophilized and preserved at −20 °C. The soluble fractions were analyzed by high-resolution size-exclusion chromatography (HRSEC) in order to determine the molecular distribution and to quantify the proteins obtained from samples [27]. The lyophilized samples were resuspended in 0.4 µL of 300 mM ammonium acetate and centrifuged (12 000 × g for 5 min). The supernatant was filtered through 0.22 µm acetate cellulose filters (Millipore GSE), and 100 µL of supernatant was then injected into the chromatographic system. The analyses were carried out 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 a 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 mM 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 between 0 and 1 mg/mL.

Polysaccharide extraction and determination by HRSEC-RID

The samples were processed using the methodology described by [28]. Briefly, 10 mL of sample was concentrated to a final volume of 2 mL using a vacuum evaporator (Univap 148 100ECH; Progen Scientific, London, UK). Total soluble polysaccharides were precipitated by adding 10 mL of cold acidified ethanol (hydrochloric acid 0.3 M in absolute ethanol) and kept for 24 h at 4 °C. The samples were then centrifuged (10,000 × g for 15 min) and the supernatants discarded. Finally, the precipitates were dissolved in 1 mL of ultra-pure water, frozen to −20 °C and freeze-dried using a lyophiliser (Telstar LyoQuest HT40 Beijer Electronics). The soluble fractions were analyzed by high-resolution size-exclusion chromatography (HRSEC) in order to determine the molecular distribution and quantify the polysaccharides obtained from the samples [28]. The lyophilized samples were resuspended in 1 mL of 50 mM ammonium formate and filtered through 0.22 µm acetate cellulose filters (Merck Millipore, Darmstadt, Germany). Then, 100 µL was injected into the chromatographic system. The analyses were carried out in an HPLC Agilent 1200 Series system (Agilent Technologies Inc., Santa Clara, USA) with a refractive index detector. 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, Japan). The mobile phase consisted of an aqueous solution of 50 mM ammonium formate applied with a constant flow of 0.6 mL/min for 60 min, and the cell RID temperature was 35 °C. The molecular weight distribution of the wine fractions was followed by calibration with a pullulan calibration kit Shodex P-82 (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 (Barcelona, Spain) and four dextrans (BioChemika; 12, 25, 50 and 80 kDa) purchased from Fluka (St. Louis, MO, USA). 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, Saint Louis, MO, USA) in the range between 0 and 2 g/L (r2 > 0.99).

Sensory analyses

All sensory analyses were performed in the tasting room of the Faculty of Enology in Tarragona (Universitat Rovira i Virgili), which was designed according to UNE 87004.197. Tasting was carried out with the ISO official tasting glasses (ISO 3591.1977). To evaluate the organoleptic characteristics of the various samples, all the sparkling wines were tasted by a group of twelve expert enologists from the Rovira i Virgili University. A sensory triangle test was conducted according to UNE ISO 4120.1983 in order to compare sparkling wine from conventional inoculation with sparkling wine from sequential inoculation.

The main objective was to determine whether the tasters were able to recognize which sparkling wine was different. The second objective was to determine which sparkling wine was preferred by the panelists who had correctly identified the various wines.

Statistics

All physical and chemical data are expressed as the arithmetic average ± the standard deviation from three replicates. One-factor analysis of variance (ANOVA) and Tukey's test were carried out with SPSS software (SPSS Inc., Chicago, Illinois, USA). The level of significance of the sensory triangle test was determined by Jackson's method [29].

Results and discussion

Table 1 shows the general parameters of the base wines and the sparkling wines. As expected, the ethanol content of the sparkling wines was around 1.3 % greater than that of the base wines. This increase corresponds to the transformation of the added sugar (22 g/L) into ethanol with a transformation ratio of 16.9 g/L by ethanol degree. Since both base wines had similar ethanol contents, their corresponding sparkling wines also had similar values. In general, the titratable acidity of sparkling wines was significantly lower than in the base wines and their pH was significantly higher. These data are also logical because the increase in ethanol causes a decrease in hydrogen tartrate solubility [30]. Volatile acidity and glycerol concentration increased after the second fermentation and were significantly higher in the sparkling wines. In general, these changes between base wines and sparkling wines are in agreement with previously published data [7, 31].

In our previous study, we observed that sequential inoculation with *T. delbrueckii* and *S. cerevisiae* produced base wines with significantly higher glycerol contents and significantly lower volatile acidities than those obtained by conventional inoculation. These data are in agreement with those of other studies that showed that sequential inoculation with *T. delbrueckii* and *S. cerevisiae* produced more glycerol and less acetic acid than conventional inoculation with *S. cerevisiae* [14, 20]. However, after the second fermentation, the volatile acidity was identical in both sparkling wines. The glycerol concentration of the sparkling wine from sequential inoculation tended to be higher than that of the sparkling wine from conventional inoculation, though this difference was not significant. It appears that the *prise de mousse* cushioned these effects.

Figure 1 shows the foam properties of the base and sparkling wines. As expected, the maximal height of the foam (HM) was significantly lower in both sparkling wines than in their corresponding base wines. This diminution, which has previously been reported [7, 31], can be attributed to the increase in ethanol content, which exerts a negative effect on wine foamability [32], and to the absorption of proteins by bentonite added as a riddling agent [11]. In

Table 1 Influence of sequential inoculation (*Torulaspora delbrueckii*/*Saccharomyces* cerevisiae) in the first fermentation on the general parameters of base wine and sparkling wine

Parameter	Base wine						Sparkling wine					
	Conventional			Sequential			Conventional			Sequential		
Ethanol (%)	10.7 ± 0.1	A	α	10.7 ± 0.1	A	α	12.0 ± 0.1	A	β	11.9 ± 0.1	A	β
TA (g/L)	5.68 ± 0.01	A	β	5.60 ± 0.02	A	β	5.25 ± 0.06	A	α	5.15 ± 0.06	A	α
pH	2.81 ± 0.01	A	α	2.80 ± 0.01	A	α	3.03 ± 0.01	A	β	3.02 ± 0.01	A	β
VA (g/L)	0.18 ± 0.01	B	α	0.12 ± 0.02	A	α	0.22 ± 0.02	A	β	0.22 ± 0.04	A	β
Glycerol (g/L)	4.70 ± 0.30	A	α	5.30 ± 0.14	B	α	5.80 ± 0.36	A	β	6.37 ± 0.54	A	β

All data are expressed as the arithmetic mean of three replicates ± standard deviation. *TA* titratable acidity expressed as g of tartaric acid/L, *VA* volatile acidity expressed as g of acetic acid/L. Different Latin capital letters indicate the existence of statistically significant differences ($p < 0.05$) between conventional and sequential inoculations. Different Greek letters indicate the existence of statistically significant differences ($p < 0.05$) between base wines and its corresponding sparkling wines

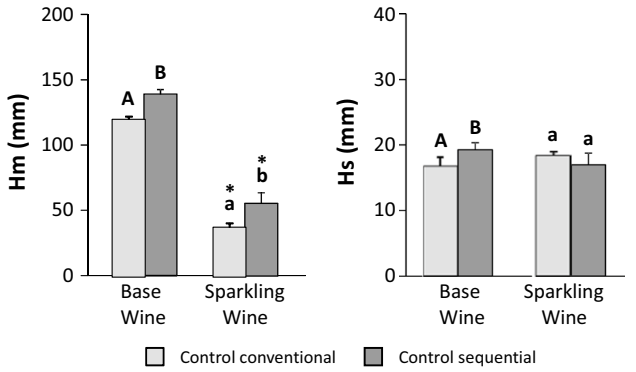

Fig. 1 Influence of sequential inoculation (*Torulaspora delbrueckii*/*Saccharomyces cerevisiae*) in the first fermentation on the foam properties of base wine and sparkling wine. All data are expressed as the arithmetic mean of three replicates ± standard deviation. *Hm* maximal height of the foam, *Hs* stable height of the foam. *Different capital letters* indicate the existence of statistically significant differences ($p < 0.05$) between base wines. *Different lowercase letters* indicate the existence of statistically significant differences ($p < 0.05$) between sparkling wines. *Asterisk* indicates the existence of statistically significant differences ($p < 0.05$) between base wines and sparkling wines

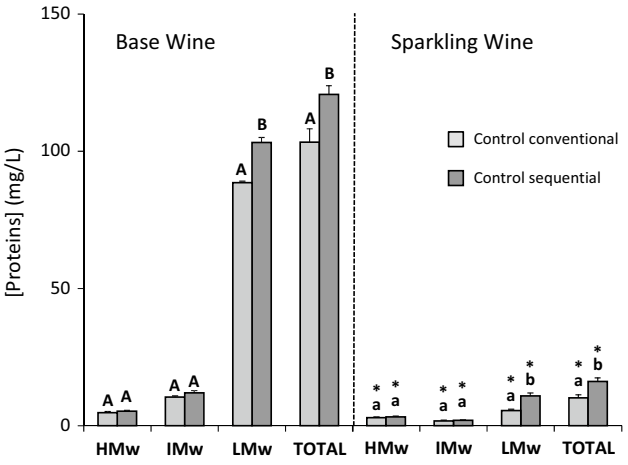

Fig. 2 Influence of sequential inoculation (*Torulaspora delbrueckii*/*Saccharomyces cerevisiae*) in the first fermentation on the protein fraction of base wines and sparkling wines. All data are expressed as the arithmetic mean of three replicates ± standard deviation. *HMw* high molecular weight protein fraction (Mw > 80 kDa), *IMw* intermediate molecular weight protein fraction (80 kDa > Mw > 60 kDa), *LMw* low molecular weight protein fraction (Mw < 60 kDa). *Different capital letters* indicate the existence of statistically significant differences ($p < 0.05$) between base wines. *Different lowercase letters* indicate the existence of statistically significant differences ($p < 0.05$) between sparkling wines. *Asterisk* indicates the existence of statistically significant differences ($p < 0.05$) between base wines and sparkling wines

contrast, the second fermentation did not lead to any difference in the stable height of the foam (HS). Our data therefore confirm that the *prise de mousse* decreases the maximal height of the foam.

In our previous study [24], we reported that sequential inoculation produced base wines with better foaming characteristics than conventional inoculation (significantly higher HM and HS values). Our present results confirm that HM is significantly higher in the sequential inoculation of sparkling wines than in conventional inoculation. However, we found no differences in HS. We may therefore assert that the positive effect on HM of sequential inoculation with *T. delbrueckii* and *S. cerevisiae* observed in the base wines is maintained in the sparkling wines.

Figure 2 shows the protein fractions of the base wines and the sparkling wines. As expected, the total protein contents of the base wines were far higher than those of the sparkling wines. This sharp decrease in the total protein content of the sparkling wines was significant in both (conventional and sequential) wines and all molecular weight fractions, especially the low molecular weight fraction (LMW). It has previously been reported and largely attributed to the absorption of proteins by the bentonite used as a riddling agent [11]. Also, it is probably one of the main reasons why HM is lower in sparkling wines than in their corresponding base wines.

In our previous study [24], we showed that a base wine obtained by sequential inoculation with *T. delbrueckii* and *S. cerevisiae* had a significantly higher protein concentration, especially of the low molecular weight fraction (LMW), than its corresponding control obtained by conventional inoculation with *S. cerevisiae*. After the second fermentation and 9 months of aging, the total protein of sparkling wines produced by sequential inoculation was still higher than that of conventional wines despite the decrease described above. This difference was again due to the LMW fraction. Since it has been reported that proteins have clear positive effects on foam [33], this greater concentration of the LMW protein fraction may be why sequential inoculation generates better foam properties than conventional inoculation in base wines and sparkling wines.

Figure 3 shows the polysaccharide fraction of base wines and sparkling wines. Again the concentration of polysaccharides in sparkling wines was significantly lower than in their corresponding base wines, and this was true in all molecular weight fractions. Similar results have been reported by other authors [34, 35], who have attributed this decrease to precipitation. Since ethanol decreases the solubility of some polysaccharides, the increase in ethanol concentration caused by the second fermentation may explain this phenomenon. Another possible explanation is the absorption of some polysaccharides by the bentonite used as riddling agent or even by the dead yeast cells.

No significant differences were found in total polysaccharides of base wines between conventional and sequential inoculation [24], and similar results were observed in

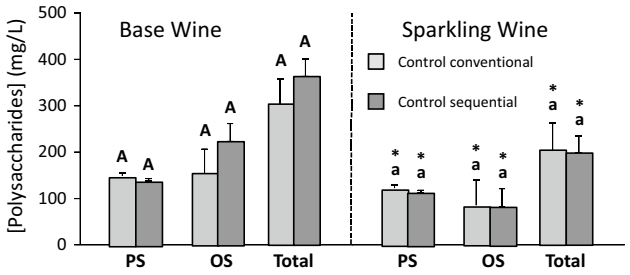

Fig. 3 Influence of sequential inoculation (*Torulaspora delbrueckii*/*Saccharomyces cerevisiae*) in the first fermentation on the polysaccharides and oligosaccharides of base wine and sparkling wine. All data are expressed as the arithmetic mean of three replicates ± standard deviation. *PS* polysaccharides (MW > 5 kDa) *OS* oligosaccharides: (MW: < 5 kDa). *Different capital letters* indicate the existence of statistically significant differences ($p < 0.05$) between base wines. *Different lowercase letters* indicate the existence of statistically significant differences ($p < 0.05$) between sparkling wines. *Asterisk* indicates the existence of statistically significant differences ($p < 0.05$) between base wines and sparkling wines

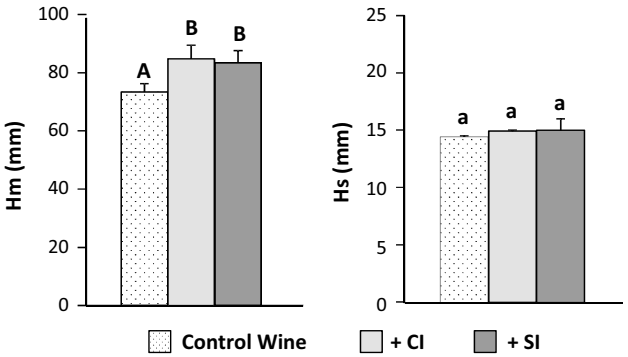

Fig. 4 Influence of supplementation with the macromolecular fraction from the different sparking wines on the wine foam parameters. All data are expressed as the arithmetic mean of three replicates ± standard deviation. *Hm* maximal height of the foam, *Hs* stable height of the foam. **+:** indicates supplementation with sparkling wine macromolecular fraction; *CI* conventional inoculation, *SI* sequential inoculation. *Different letters* indicate the existence of statistically significant differences ($p < 0.05$) between base wines

sparkling wines. In fact, all the molecular weight fractions of the base wines and sparkling wines were similar. Some strains of *T. delbrueckii* are reported to have a greater polysaccharide production capacity than *S. cerevisiae* [22, 23], but our results do not confirm this, probably because the strain of *T. delbrueckii* we used was different.

Table 2 shows the results of the sensory triangle test. Nine tasters out of twelve were able to distinguish the sparkling wine from sequential inoculation with *T. delbrueckii* and *S. cerevisiae* from the sparkling wine from conventional inoculation with *S. cerevisiae*. These data are statistically significant ($p = 0.01$) and indicate the existence of sensory differences between the two sparkling wines. Moreover, six of the nine tasters who correctly identified the different sample preferred the sparkling wine from sequential inoculation because its effervescence was more integrated and because it was generally less aggressive in the mouth. It seems, therefore, that the better foam properties measured are reflected in the sensory perception of effervescence.

Figure 4 shows the foam properties of a white wine in comparison with the same wine enriched with the macromolecular fractions of both sparkling wines—conventional (+ CI) and sequential (+ SI)—in order to determine how these colloids influence foamability. Our results indicate

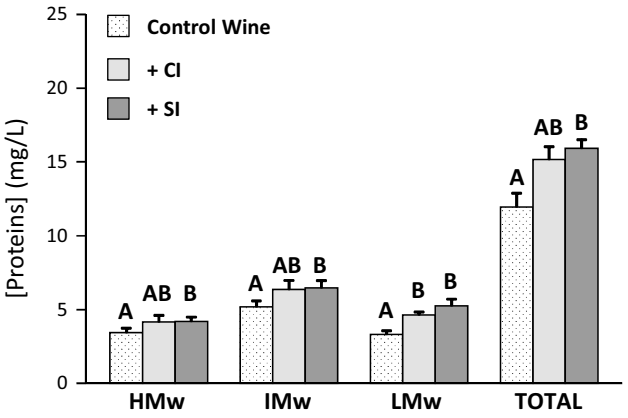

Fig. 5 Influence of king wines on the protein fraction. All data are expressed as the arithmetic mean of three replicates ± standard deviation. *HMw* high molecular weight protein fraction (Mw > 80 kDa), *IMw* intermediate molecular weight protein fraction (80 kDa > Mw > 60 kDa) and *LMw* low molecular weight protein fraction (Mw < 60 kDa). *Different letters* indicate the existence of statistically significant differences ($p < 0.05$) between base wines

Table 2 Sensorial analysis results for sparkling wines

Triangular test	Positive identifications	*p*	Preferences	
Control versus Sequential	9/12	0.01	Control 3	Sequential 6

that supplementation with the macromolecular fraction of both sparkling wines significantly increased HM in comparison with the control wine. However, no differences were observed between the + SI and + CI wines. Moreover, enriching the wine with the macromolecular fraction of any of the sparkling wines did not affect HS.

The protein fractions of the control wine and the enriched wines were analyzed (see Fig. 5). The total protein concentration tended to be higher in the enriched wines, but this increase was only significant in the case of the + SI wine. This behavior was also observed for the HMW and IMW

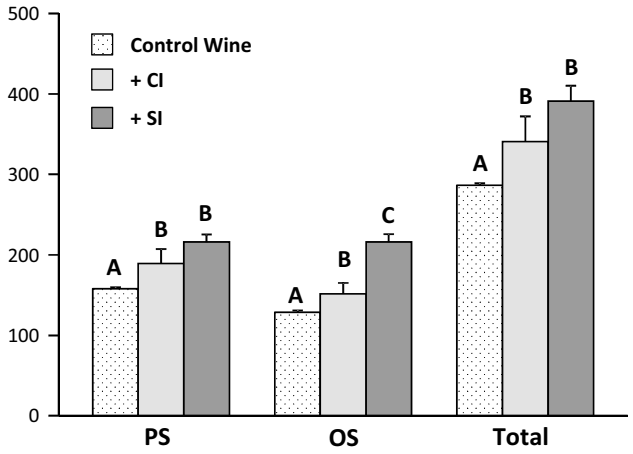

Fig. 6 Influence of supplementation with the macromolecular fraction from the different sparking wines on the polysaccharide fraction. All data are expressed as the arithmetic mean of three replicates ± standard deviation. *PS* polysaccharides (Mw > 5 kDa), *OS* oligosaccharides (Mw < 5 kDa). **+** indicates supplementation with sparkling wine macromolecular fraction. *CI* conventional inoculation, *SI* sequential inoculation. *Different letters* indicate the existence of statistically significant differences ($p < 0.05$) between base wines

protein fractions. However, the increase in LMW fraction was significant in both enriched wines. In general, these data are in agreement with the protein concentrations observed in the different sparkling wines. Since sparkling wine from sequential inoculation was richer in proteins than the sparkling wine from conventional inoculation, it is logical that the protein concentration of the wine enriched with its colloids should also be richer. However, the increase in total protein concentration in both enriched wines was lower than expected considering the protein concentration of the sparkling wines used to obtain the protein fraction for the enrichment. In fact, the recoveries were 47.5 ± 2.7 % for + CI wine and 37.0 ± 3.7 % for the + SI wine. This lower recovery, probably due to denaturation of proteins during the isolation process of the macromolecular fraction, may be the reason because the protein content of + SI wine was not significant for the + CI wine and was not reflected in its foam properties, which were similar to those of the + CI wine.

Figure 6 shows the polysaccharide fraction of the control wine and the enriched wines. In general, the total polysaccharide concentrations of both enriched wines were significantly higher than that of the control wine. These differences were found both in the polysaccharide fraction (PS) and in the oligosaccharide fraction (OS). However, as it happens with proteins, the increase in total polysaccharide concentration in both enriched wines was lower than expected considering their concentration in the original sparkling wines. Specifically, the recovery was 39.3 ± 9.3 % for + CI wine and 78.7 ± 4.9 % for + SI wine. The higher recovery observed in the isolation process of the macromolecular fraction of + SI wine is difficult to be explained, but it might be related with differences in the composition of the polysaccharides released by *T. delbrueckii* during the first fermentation. This is only an hypothesis, but a different composition of the polysaccharides released by *T. delbrueckii* in the sparkling wine obtained from sequential inoculation would justify why it was felt to be less aggressive in the sensory tasting since it has been reported that polysaccharides, oligosaccharides and mannoproteins contribute to the sensation of sweetness [23, 36]. In any case, this different recovery is probably the reason because the PS, OS and total polysaccharide concentrations tended to be higher in the + SI wine than in the + CI wine, though the concentration was only statistically significant in the case of oligosaccharides.

It can therefore be concluded that sequential inoculation with *T. delbrueckii* and *S. cerevisiae* may be useful for obtaining sparkling wines with better foaming properties. Specifically, sequential inoculation produced base wines with significantly higher maximum heights of foam (HM) than conventional inoculation, probably because autolysis of the *T. delbrueckii* cells in the base wine released higher amounts of proteins, especially of the low molecular weight fraction. This trend of higher protein concentration and better HM was maintained in the sparkling wines from sequential inoculation though, logically, both values were reduced by the *prise de mousse*.

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Compliance with ethical standards

Conflict of interest Two of the authors work for the company which commercializes the yeasts strains used in this article.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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