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Effect of yeast mannoproteins and grape polysaccharides on the growth of wine lactic acid and acetic acid bacteria

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ABSTRACT

Polysaccharides constitute one of the main groups of wine macromolecules and the difficulty in separating and purifying them has given as a result that they have been less studied than other wine macromolecules. In this study the biological activity of a number of polysaccharide fractions obtained from yeast lees, must and wine has been analysed against a large collection of both lactic acid bacteria (LAB) and acetic acid bacteria (AAB) of enological origin. Results showed that a high proportion of AAB strains (60 - 88 %) was inhibited by concentrations lower than 50 mg/l of polysaccharide fractions containing intermediate (6 – 22 kD) and small molecular weight (< 6 kD) mannoproteins and oligosaccharide fragments derived from cellulose and hemicelluloses. Results showed as well that in contrast, yeast mannoproteins in concentrations up to 200 mg/l activated the growth of 23 – 48 % of the studied LAB strains when ethanol was present in the culture broth. Specially yeast commercial mannoproteins of intermediate molecular weight were active in increasing *Oenococcus oeni* growth (81.5 % of the studied *O. oeni* strains) in presence of ethanol in the culture broth. These effects of wine polysaccharides on bacterial growth provide novel and useful information for microbiological control of wines and winemaking biotechnology.

KEYWORDS: mannoproteins, wine polysaccharides, lactic acid bacteria, acetic acid bacteria.

INTRODUCTION

Polysaccharides are one of the main groups of wine macromolecules. Wine polysaccharides are grouped in two families according to their origin: those originating from grape primary cell walls, and those released by microorganisms, which include yeast and bacteria, and fungi when grapes are infected. According to their acidity and protein content, polysaccharides can be subsequently subgrouped. Polysaccharides from grape berries have pectin as one of their main constituent, and neutral pectic substances mainly comprise type II arabinogalactans (AG) and arabinogalactan-proteins (AGP), which represent more than 40% of total red wine polysaccharides *(1)*. The second most abundant family of polysaccharides in red wine is that of mannoproteins (MP) *(1,2)*. The origin of these macromolecules is yeast cell walls and they are released from yeast cells in the early stages of fermentation and during later stages when wine ageing is performed in contact with lees *(3)*. Wine mannoproteins have highly variable sizes and are constituted by mannans and less than 10 % protein *(1,4)*. These polysaccharides, which can account for up to 50% of the cell wall dry mass of *Saccharomyces cerevisiae*, are located in the outermost layer of the cell wall, where they are connected to a matrix amorphous β-1,3 glucan by covalently bonds *(5)*. Grape berry acid pectic polysaccharides constitute the third most abundant group of polysaccharides in wine, they are characterised by a high proportion of galacturonic acid, and they include homogalacturonans (HG), rhamnogalacturonans I (RG-I) and rhamnogalacturonans II (RG-II) *(1)*.

The difficulty in separating and purifying all these wine polysaccharides has given as a result that they have been less studied than polyphenolic compounds, the other major group of macromolecules present in wine. Thus, wine and grape polyphenolic

compounds have been shown to inhibit a number of enological lactic acid bacteria (LAB) *(6-8)* and of pathogenic bacteria from a variety of origins *(9-15)*.

During wine-making the microbiota associated to the process evolves and is in a continuous dynamic equilibrium. Yeast is the predominant microorganism during alcoholic fermentation, and once it is finished, LAB take the lead and carry out the secondary fermentation, named malolactic fermentation (MLF). LAB reach populations around 10^6 CFU/ml, and essentially the species *Oenococcus oeni* is the one that imposes and conducts the transformations during MLF. Nowadays, MLF is recommended for red wines, especially those wines of quality that are to be submitted to the ageing process *(16)*. Acetic acid bacteria (AAB) are ubiquitous bacteria, they are strict aerobes that require oxygen for their growth, and they are present during the whole process of wine-making but are kept in a latent state without proliferation, mainly due to the quite strict anaerobic conditions under which the winemaker maintains wine during the whole process (*17*).

Interaction of wine polysaccharides with the natural microbiota of musts and wines has not been studied in depth. The presence of polysaccharides in must and wine might have as a consequence either activation or inactivation of bacterial growth *(18)*, and it may be as well a two-way interaction, i.e., microorganisms may degrade wine polysaccharides, and thus decrease total polysaccharide content, and may as well synthesise new polysaccharides *(19)* that are released into wine.

The aim of this paper was to investigate the biological activity of a number of polysaccharide fractions obtained from yeast lees, must and wine against a large collection of both LAB and AAB isolated from wines, musts and wine vinegars. This collection of bacteria contained both wine spoilage species with potential to cause wine

organoleptic and hygienic alterations, and beneficial strains able to conduct a correct MLF in wines. Additionally, the effect of two polyphenolic compounds of wine: malvidine, as representative molecule of red wine anthocyanins, and catechin, as representative molecule of tannins, was investigated.

MATERIALS AND METHODS

Bacteria strains. The following bacteria strains were used in this study: 65 LAB (27 *Oenococcus*, 30 *Lactobacillus*, 6 *Pediococcus*, 1 *Leuconostoc*, 1 *Lactococcus*), 25 AAB strains (7 *Gluconobacter*, 11 *Acetobacter*, and 7 *Gluconacetobacter*). Most of the strains were isolated from wine and vinegar (strains belonging to the microbial culture collection of the University of La Rioja), and Table 1 shows the origins and species of all the strains of this study.

Culture and growth conditions. LAB except *O. oeni* were cultivated for 48 h onto MRS agar plates (Scharlau Chemie S.A., Barcelona, Spain) at 30ºC in an air atmosphere containing 5% CO_2. *O. oeni* was cultivated for 4-6 days onto MLO-agar plates (35 g/l MLO, 15 g/l agar, 1 ml/l polysorbate 80, 100 ml/l tomato serum) (Scharlau Chemie S.A) at 30ºC under strict anaerobic conditions (anaerobic system BR038B, Oxoid Ltd., Basingstoke, England) (7-10 % final CO_2 concentration). AAB were cultivated for 48 h onto mannitol agar plates [25 g/l n-manitol (Panreac Quimica S.A., Barcelona, Spain), 5 g/l yeast extract (Scharlau Chemie S. A) and 3 g/l peptone (Becton, Dickinson Co., Le pont de Claix, France).

Reagents and equipments: All reagents were analytical grade unless otherwise stated. L-fucose, L-rhamnose, 2-*O*-methyl D-xylose, L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose and Kdo (3-deoxy octulosonic acid) were supplied by Sigma (Beerse, Belgium), D-apiose was obtained from Omicrom (South Bend, IN), and D-

galacturonic acid, D-glucuronic acid and myo-inositol (internal standard) were obtained from Fluka (Buch, Switzerland). Ethanol 96% (v/v), hexane and acetyl chloride were supplied by Scharlab (Barcelona, Spain), hydrochloric acid 37% was purchased from Carlo Erba (Rodano, Milan, Italy), and dried methanol, pyridine, hexamethyldisilazane and trimethylclorosilane were obtained by Merck (Darmstadt, Germany). Ammonium formiate of HPLC grade supplied by Fluka (Buch, Switzerland) and MilliQ deionised water (Millipore, Molsheim, France) were used. A pullulan calibration kit (Shodex P-82) was obtained from Waters (Barcelona, Spain). The enzymes used for the lees (β-glucanases and pectinases) were supplied by Novozymes Biopharma (Theberton, Australia). Commercial mannoproteins were purchased from AEB S.p.A. (Brescia, Italy).

High-resolution size-exclusion chromatography (HRSEC) was performed using a modular 1100 Agilent liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with one G1311A quaternary pump, an on-line G1379A degasser, a G1316A column oven, a G1362 refractive index detector, a manual injector (Rheodyne, CA, USA), a Gilson fraction collector (Middletown, WI, USA), and controlled by the Chemstation Agilent software. The gas chromatography (GC) system controlled by the Chemstation software consisted of an HP5890 Series II gas chromatograph (Hewlett-Packard, USA) coupled to a flame ionization detector (FID).

Production of lysated yeast lees and wine elaboration. Yeast lees were obtained from wine produced at the CVNE winery of the Qualified Origin Denomination Rioja (D.O.Ca Rioja). The wine was made from *Tempranillo* grapes using traditional vinification techniques. After racking of the red wine after malolactic fermentation, the lees deposited in the bottom of the vat were recovered in a proportion of 80:20 (v/v) lees and wine, and they were then treated with tartaric acid (2.5 g/l) and corrected to 40 mg/l

of free SO_2. Then, the lees were distributed in used barrels and treated with 15 g/Hl of a commercial mixture of pectinases and β-glucanases. All the barrels were rotated daily and kept at a temperature of 10ºC. The free SO_2 was analyzed regularly and kept between 35 and 40 mg/l. After 30 days, lysated lees were recovered in a proportion of 80:20 (v/v) lees and wine, and microscopic inspection and counting in a Neubauer chamber revealed a population of 3 x 10^8 lysed cells/ml. This sample of lysated lees was submitted to the polysaccharide extraction method described ~~below~~ in the following section.

Tempranillo grapes of D. O. Ca Rioja were used for wine elaborations in the wine cellar of the University of La Rioja. Grapes were destemmed, crushed and fermented into 100 l stainless steel tanks. The prefermentation process went on for 6 h at 18 ± 1 ºC; the fermentation–maceration process was carried out at a maximum temperature of 28 ± 2 ºC and lasted for 10 days. Postfermentative maceration went on for 4 days at 24 ± 1 ºC and wines were run off. Samples were taken during the first stages of alcoholic fermentation (must sample) and after the postfermentative maceration (wine samples). Both samples were submitted to the polysaccharide extraction method described below ~~in the following section~~.

Extraction of ~~total~~ polysaccharides from lees, must and wine samples. Total polysaccharides were extracted from the lysated lees, must and wine samples following the method described by Ayestarán and col. *(20)*. Samples were centrifuged (14,000 x g for 5 min) using a RC-5B Sorvall refrigerated centrifuge (Du Pont, BH, Germany) and supernatants were then concentrated under reduced pressure at 34ºC. Polysaccharides were precipitated by adding cold acidified ethanol (96 % ethanol, containing HCl 0.3 M) and kept for 18 h at 4 ºC. Thereafter, samples were centrifuged (14,000 x g for 20 min), the supernatants discarded, and the pellets washed several times with 96% ethanol

to remove interference materials. Polysaccharide precipitates were dissolved in ultrapure water and freeze-dried using a Virtis freeze drying (New York, USA).

In order to obtain different polysaccharide fractions, lees, must and wine polysaccharide precipitates were subjected to high-resolution size-exclusion chromatography (HRSEC) on a Superdex-75 HR (1.3 x 30 cm) column (Pharmacia, Sweden) (exclusion size = 3 kD) equilibrated at 0.6 ml/min in 30 mM ammonium formiate, pH 5.8 as previously described *(20)*. ~~and thus, small molecules (< 3 kD) were discarded~~ The peaks obtained were collected in different fractions (S1, S2 and S3) according to their molecular weights: S1 fraction (50-400 kD), S2 fraction (6-22 kD) and S3 fraction (< 6 kD). The eluted fractions were freeze-dried, redissolved in water, and freeze-dried again four times to remove ammonium salt. Each sample was injected at least 40 times in order to obtain enough freeze-dried quantities for further analyses.

~~The eluted fractions were freeze-dried, redissolved in water, freeze-dried again four times to remove ammonium salt, and finally they were pooled together. These desalted total polysaccharide pooled samples from lees, must and wine were named as M1, P1 and P2 respectively~~.

~~Commercial mannoproteins were purchased from AEB S.p.A. (Brescia, Italy) and two types were assayed: those named as M2 (mannoproteins of intermediate molecular weight), and those named as M3 (mannoproteins of low molecular weight)~~.

~~The composition analysis of these five samples (M1, M2, M3, P1 and P2) was performed as indicated in the next section, and they were all tested in the bacterial growth inhibition microtiter assays as well as the fractionation peaks (S1, S2 and S3) described below~~.

Polysaccharide fractionation and composition analysis. Lees, must and wine total polysaccharide precipitates and commercial mannoproteins M2 and M3 were submitted to further fractionation by high resolution size exclusion chromatography (HRSEC) on the Superdex-75 HR (1.3 x 30 cm) column (Pharmacia, Sweden) equilibrated at 0.6 ml/min in 30 mM ammonium formiate, pH 5.8. Chromatographic separation was carried out at room temperature on an Agilent modular 1100 liquid chromatograph (Waldbronn, Germany) connected to a G1362 refractive index detector as previously described *(19)*. Three peaks with different elution times were obtained (named S1, S2 and S3) from each polysaccharide sample. These isolated peaks were freeze-dried, redissolved in water, and freeze-dried again four times to remove ammonium salt. Each sample was injected at least 40 times in order to obtain enough freeze-dried quantities for further analyses. The molecular weight distribution of these polysaccharide fractions was determined by calibration with narrow pullulan molecular weight standards (Shodex P-82, Waters, Barcelona, Spain): P-5, Mw = 5.9 kD; P-10, Mw = 11.8 kD and P-50, Mw = 47.3 kD. The apparent molecular weights were deduced from the calibration equation $\log M_w = 11.188 - 0.403\ t_R$ (t_R = column retention time at peak maximum, and r^2 = 0.999).

The monosaccharide composition of each sample and fraction obtained from lees, must and wine samples was analysed by gas chromatography with flame ionization detector (GC-FID) after acidic methanolysis and derivatization as previously described *(20)*. Different standard carbohydrates were also derivatized and analysed by GC-FID in order to obtain patterns for identification and standard calibration curves. Polysaccharide composition of the fractions was estimated from the concentration of individual glycosyl residues that were characteristic of well-defined wine

polysaccharides, as previously described *(20, 21)*. ~~Polysaccharide composition of all samples is shown in Table 2.~~

Samples assayed for microbiological activity. Two types of commercial mannoproteins were directly assayed: those named as M1 (mannoproteins of intermediate molecular weight), and those named as M2 (mannoproteins of low molecular weight). The pooled fractions S1+S2+S3 obtained from lees sample (named L), grape must sample (named G) and wine sample (named W) were tested. In addition, isolated fractions of different polysaccharide composition were also assayed. Therefore, fraction S1 (named S1) from the must sample and fraction S2 (named S2) and fraction S3 (named S3) from the wine samples were also tested. Polysaccharide composition of these samples is shown in Table 2.

Growth inhibitory activity. Bacteria growth inhibitory activity of polysaccharide samples was determined by calculating the minimal inhibitory concentration (MIC) in the microtiter dilution assay *(22)* as follows. MRS broth was used for LAB except *O. oeni*, for which MLO broth was used, and mannitol broth was used for AAB. Microtiter plates were incubated at 30ºC for 48 h, after which bacterial growth was measured by optical density at 655 nm in a microtiter reader (Model 45, Bio-Rad Laboratories, Hercules, CA). MIC was defined as the smallest concentration of sample needed to inhibit 50% of the bacterial growth after 48 h of incubation. Positive and negative controls were included in all assays. All freeze-dried polysaccharide samples described above were dissolved in sterile ultrapure water and used in the microtiter assay. Samples were tested in serial double dilutions starting with concentrations that can be normally found in enological conditions *(2, 23)*: G and S1 from 300 to 0.145 mg/l; W from 800 to

0.39 mg/l and S2 and S3 from 100 to 0.045 mg/l. M1 and M2 were tested in serial double dilutions starting with amounts usually recommended by the manufacturers: from 200 to 0.095 mg/l.

Two wine polyphenols were also assayed by the microtiter dilution method: malvidin (Extrasynthese, Lyon, France), as a representative molecule of red wine anthocyanins, and catechin (Extrasynthese), as the representative molecule of tannins. Malvidin was assayed in the range concentration from 700 to 0.34 mg/l and catechin from 8557 to 4.17 mg/l. Both ranges include the average concentrations of these polyphenols that can be normally found in red wines *(8)*.

Ethanol combined effect on bacterial growth. The combined effect of ethanol and polysaccharides on bacterial growth was also investigated. Ethanol concentrations of 3% and 6% in the microtiter assays (included as well in control samples) were used for AAB and LAB respectively. In those experiments performed with LAB where bacterial growth activation was observed, minimal activating concentration of the polysaccharide sample was defined as the highest dilution that rendered 50% increase of bacterial growth after 24 h incubation in the case of *O. oeni* and AAB, and after 12 h in the case of other LAB strains. The combined effect of ethanol and either malvidin or catechin under the same experimental conditions as described for polysaccharides was also investigated for AAB and LAB.

Statistical procedures: Microbiological assays were performed in duplicate. Significant differences between samples were analyzed with the SPSS 15.0 program for Microsoft Windows (SPSS Inc., Chicago, IL) by the nonparametric U Mann–Whitney test.

RESULTS AND DISCUSSION

Bacterial growth inhibitory effect. Fig 1 shows the MIC values against LAB and AAB strains of the total polysaccharide extract from yeast lees (sample L). As shown in this figure, all LAB strains of this study (including *O. oeni* strains) were not inhibited by the yeast polysaccharide extract (MIC values >200 mg/l), whose composition was mainly yeast mannoproteins (75.1 %) (Table 2). Fig 1 shows that in contrast to LAB strains, most AAB strains (88 %) were inhibited ($p<0.001$) by 50 mg/l or lower concentrations of this polysaccharide extract from yeast lees (sample L) and Fig 2 shows that the most susceptible AAB strains to the yeast mannoprotein-rich extract were those of the genus *Gluconacetobacter*, followed by *Acetobacter* and *Gluconobacter*. When the commercial mannoproteins M1 and M2 were assayed separately, similar results were obtained (Fig 3) in that AAB growth was inhibited ($p<0.001$) by 50 mg/l or lower concentrations of both types of commercial mannoproteins (M1 and M2) for 76 % of the studied AAB strains. *Gluconacetobacter* continued showing the highest susceptibility to both commercial mannoprotein samples, and all LAB strains of our study were not affected by the presence of these mannoproteins in the growth medium (data not shown). These results indicate that yeast mannoproteins, currently used as enological tools to stabilise wine colour and sensorial properties *(3)* can also prevent AAB growth and contribute to microbiological control during winemaking. It is worth noting that both commercial samples were rich in low molecular weight mannoproteins (< 6 kD), and that the L sample obtained from lees contained mannoproteins of a range of molecular weights (Table 2). To clarify which the active molecules in inhibiting AAB growth were, the next experiments were carried out with the other polysaccharide extracts and fractions of this study.

Fig 4A shows the MIC values of the grape must polysaccharide extract (sample G) against AAB strains and indicates that all tested AAB except five strains (80 % of the studied AAB strains) were sensitive to 300 mg/l of this polysaccharide extract ($p<0.001$), which is the concentration that can be normally found in grape musts (*2, 23*), whereas it had no effect on any of the LAB strains of this study (data not shown). This Fig 4A shows as well that *Gluconactobacter* strains were more sensitive to the must polysaccharide extract than AAB of the other genera. As shown in Table 2, this polysaccharide extract contained mainly glucosyl oligosaccharides derived from cellulose and hemicellulose fragments (60 %). In contrast, the total polysaccharide extract obtained from wine after alcoholic fermentation (sample W) showed no major inhibitory activity against AAB, and most strains (76 %) remained resistant (MIC > 800 mg/l) to this polysaccharide extract (Fig 4B) that contained high molecular weight arabinogalactan-proteins and mannoproteins (50 – 400 kD molecular weight) as its major components (Table 2). All these results indicated that the active molecules in inhibiting AAB growth were intermediate (6 – 22 kD) and low molecular weight (< 6 kD) mannoproteins as well as small oligosaccharides derived from cellulose and hemicelluloses that were only present in the polysaccharide extract from the initial grape must and that disappeared during wine fermentation *(2)*, which could be due either to their consumption by the fermenting yeast, or most probably, to precipitation caused by the ethanol formed during the alcoholic fermentation.

Subsequent polysaccharide fractionation peaks (samples S1, S2 and S3) were assayed separately by the microtiter dilution method: S1 fraction (high molecular weight polysaccharides, average value = 105 kD), S2 fraction (intermediate molecular weight polysaccharides, average value = 11.8 kD) and S3 fraction (low molecular weight polysaccharides, < 6 kD). Results showed that S1 fraction of high molecular weight

polysaccharides, which consisted of a mixture of large arabinogalactan-proteins and mannoproteins (Table 2), exerted no inhibitory effect (MIC ≥ 150 mg/l) on the growth of 72 % of the studied AAB strains (Fig 4C), and samples of intermediate (S2, 11.8 kD average molecular weight value) and low (S3, molecular weight < 6 kD) molecular weight polysaccharides retained their inhibitory effect (MIC ≤ 12.5 mg/l) on the growth of 72 % of the studied AAB strains (Fig 5). As shown in Table 2, these active samples (S2 and S3) contained mannoproteins of intermediate molecular weight (sample S2) and their corresponding oligosaccharides of low molecular weight (< 6 kD) (sample S3), and as indicated in Fig 5, they showed inhibitory activity against AAB strains ($p < 0.001$) that were also sensitive to the commercial mannoproteins (samples M1, M2) or to the yeast lees extract (sample L).

None of the studied polysaccharide samples (shown in Table 2) showed inhibitory effect on the growth of the LAB strains (data not shown).

Fig 6 shows the effect on AAB growth of catechin, as the representative molecule of wine tannins, and that ten strains were inhibited by very low concentrations of catechin (< 4.2 mg/l), much lower concentrations than the normal content (10 – 400 mg/l) found in red wines *(13)*, whereas 14 strains of our collection were not inhibited even by a high concentration of this molecule (≥2,000 mg/l). These results indicate that catechin inhibition of AAB growth is strain dependent and that bacterial response is highly polarised, in that either cells are highly resistant, or highly sensitive to catechin. To our knowledge, this is the first report on the effects of catechin on AAB growth. LAB strains of our collection showed no growth effect in presence of catechin (data not shown) in spite of the high concentrations (1 – 8 g/l) that were used in the assay conditions. Similarly to our results with our collection of LAB strains, a number of studies had reported no effect of catechin on LAB growth *(7-8,24-25)*, although

Reguant and col. *(24)* reported one *O. oeni* strain whose growth was activated by catechin, and Alberto and col. *(26-27)* and Hervert-Hernández and col. *(28)* reported two LAB strains of the genus *Lactobacillus* that were able to metabolise catechin and some other grape pomace polyphenols, and thus activated their growth.

Ethanol combined effect on bacterial growth. When the microtiter assay of mannoprotein samples (M1, M2 and L) was performed in presence of subinhibitory concentrations of ethanol (6 % for LAB) as described in methods section, results did not show any inhibitory effect but on the contrary, they showed growth activation of LAB strains, as indicated in Table 3. In presence of mannoprotein samples (M1, M2 and L) and 6 % ethanol, LAB cells increased their growth (> 50 % increase in the microtiter assay) when compared with control cells grown in absence of the mannoprotein sample. This activation was observed with a high number of LAB strains: 31 strains (48 %) were activated by 200 mg/l or lower concentrations of M1 sample, 17 strains (26 %) were activated by M2 and 15 strains (23 %) were activated by L (Table 3), whereas this activation effect was not observed with any of the AAB strains of our study (data not shown). Table 3 shows that 22 *O. oeni* strains (81.5 % of total *O. oeni*) were activated by 200 mg/l or lower concentrations of commercial mannoproteins of intermediate molecular weight (M1), and moreover, seven of these strains were activated as well by the commercial mannnoproteins of low molecular weight (M2). It is worth noting that the LAB strains of our collection that were activated by the mannoprotein samples were those of species (*O. oeni, Lactobacillus plantarum*) that contribute positively during MLF to wine sensorial properties. Early studies had reported a correlation between the liberation of yeast cell wall macromolecules during alcoholic fermentation with an increase of LAB growth *(29, 30)*. Nevertheless, those studies were performed with a reduced number of *O. oeni* isolates and the observed effect could be due in part to the

adsorption of the medium chain fatty acids synthesised by yeast. Fatty acids have been long shown to inhibit bacterial growth and, therefore, their removal by adsorption by yeast cell walls would improve bacterial growth. Our results show that there is a positive interaction between some LAB strains and yeast mannoproteins in presence of ethanol and in absence of other interfering factors such as cell membranes or fatty acids. Our results show as well that this activation is not species dependent but strain dependent, and that out of 65 studied LAB strains, from 15 to 31 strains were activated by yeast mannoprotein-rich extracts only when ethanol (6 % concentration) was present in the growth medium. Some LAB strains had been reported to be able to hydrolyse polysaccharides and, thus, to enhance the nutritional content of the medium and to increase their growth rate *(19, 31)*. It is important to note that in the case of our LAB strains mannoprotein samples increased bacterial growth exclusively in presence of ethanol, i.e. this positive interaction occurred only when there was a factor of stress for LAB survival. No effect on the growth of LAB or AAB was observed with any of the other polysaccharide samples of our study or with catechin in combination with ethanol.

Regarding the effect on bacterial growth of the molecule representative of the anthocyanin family of wines, malvidin, our results showed that alone it had no effect on bacterial growth, either on LAB, or on AAB (data not shown) and that in presence of 6 % ethanol in the growth medium, malvidin activated the growth of a number of LAB strains (34 out of the total 65 LAB strains of this study) as shown in Table 3. This result indicates that, as in the case of yeast mannoproteins (those of molecular weights < 6 and up to 22 kD), malvidin exerts a protection against the effect of ethanol in the medium. Under our lab experimental conditions, LAB strains grew less in presence of 6% ethanol than in its absence, and when the activating molecule was present (malvidin) cell growth was activated. It is important to note that all the LAB strains of this study were

of enological origin (grape, must and wine) and therefore, they had been previously in contact with grape anthocyanins and were able to grow in presence of this type of molecules. Further studies should be performed in order to clarify whether this protective effect against ethanol is exerted at the membrane level of bacterial cells.

In summary, this work reports a complete study of the effect of must and wine polysaccharides, which include the family of mannoproteins synthesised by fermenting yeast, on the growth of a wide collection of 90 bacterial strains of enological origin (grape, must, wine and vinegar). Results show important differences between LAB and AAB behaviour and provide novel and useful information for future and new applications of yeast mannoproteins in winemaking biotechnology and microbiological control.

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Table 1. Bacteria strains used in this study

Microorganism (number of strains)	Species	Number of strains	Strains	Source
LAB(n=65)	*Lactobacillus hilgardii*	1	J81	Wine
	Lactobacillus paracasei	1	J52	Wine
	Lactococcus lactis	1	C653	
	Lactobacillus plantarum	28	J21 J23 J36 I3 V6 E3 E14	Must
			Y17	Must
			J39 J51 J53 J55 J56 J58	Wine
			J59 J61 J62 J63 J65 J70	Wine
			J71 J73 J77 J78 T53 T60	Wine
			E8	Wine
	Leuconostoc mesenteroides	1	J48	Wine
	Pediococcus acidilactici	1	C652	
	Pediococcus parvulus	1	J103	Wine
	Pediococcus pentosaceus	4	J27 J29	Grape
			J40	Wine
			C531	
	Oenococcus oeni	27	IS1 IS16 IS21 IS24 IS 27	Wine
			IS44 IS45 IS46 IS47 IS 48	Wine
			IS51 IS53 IS63 IS 73 IS75	Wine
			IS129 IS144 IS 151 IS154	Wine
			IS 155 IS 159 IS186 IS189	Wine
			IS 196 IS 205 IS 209 IS210	Wine
AAB (n=25)	*Acetobacter aceti*	1	CECT 298	CECT

Acetobacter pasteurianus	7	CECT474	CECT
		IS242 IS260 IS286 IS282	Wine
		R28 R30	Cider vinegar
Acetobacter orleanensis	3	IS291 IS293 IS294	Wine
Gluconobacter oxydans ssp suboxydans	1	CECT 360	CECT
Gluconobacter oxydans	6	V3 I7	Must
		I38 I39 IS262B IS283B	Wine
Gluconacetobacter europaeus	5	R29	Wine vinegar
		R40	Cider Vinegar
		R68	Wine vinegar
		R71 R78	Spirit vinegar
Gluconacetobacter xylinus	2	R35 R46	Cider vinegar

CECT: Spanish collection of type cultures. LAB: lactic acid bacteria AAB: acetic acid bacteria.

Table 2. Polysaccharide composition of the commercial mannoproteins and yeast lees, must and wine polysaccharide samples of this study

		Polysaccharide composition (%)*									
		50-400 kD		6-22 kD			< 6 kD				
Sample	Polysaccharide Origin	AGP	MP	AGP	MP	RG-II dimers	AGP oligosaccharides	MP oligosaccharides	RG-II monomers	HG and RG oligosaccharides	Glucosyl oligosaccharides
L	Lysated Lees	8.02	26.7	8.2	27.3	3.07	5.7	21.1		0.75	
M1	Commercial mannoproteins		11.7		5.53			56.7			
M2	Commercial mannoproteins		7.4		2.18			64.05			
G	Grape must	27	5	2	0.4	0.4	2.67	0.89		0.7	60
W	Wine	32	25	12	8	12	5.29	3.63	1	2	
S1	Grape must	78	22								
S2	Wine			38	24	38					
S3	Wine						46	29	13	13	

* From 78 to 95% of total monosaccharides.
AGP: arabinogalactan-proteins.
MP: mannoproteins.
RG-II dimers: rhamnogalacturonan-II dimers.
AGP oligosaccharides: fragments of arabinogalactan-proteins of less than 6 kD.
MP oligosaccharides: fragments of mannoproteins of less than 6 kD.
RG-II monomers: rhamnogalacturonan-II monomers of less than 6 kD.
HG and RG oligosaccharides: homo- and rhamnogalacturonans with molecular weights smaller than 6 kD.
Glucosyl oligosaccharides: fragments of celluloses and hemicelluloses with molecular weights smaller than 6 kD.

Table 3. Bacterial growth activation by yeast mannoprotein samples L, M1 and M2) and malvidine in presence of ethanol in the growth medium.

Sample	% Ethanol	Type of bacteria	Minimal activating concentration[1] (mg/l)	number of strains
Yeast lees total polysaccharide extract (L)	6%	LAB*	100	1
			200	8
			No activation	29
	6%	*O. oeni*	6.25	1
			200	5
			No activation	21
	3%	AAB	No activation	All strains (25)
Commercial mannoproteins of intermediate molecular weight (M1)	6%	LAB*	3,17	2
			12,5	1
			100	2
			200	4
			No activation	29
	6%	*O. oeni*	12,5	1
			100	3
			200	18
			No activation	5
	3%	AAB	No activation	All strains (25)
Commercial mannoproteins of low molecular weight (M2)	6%	LAB*	6,25	1
			50	2
			100	1
			200	6
			No activation	28
	6%	*O. oeni*	50	1
			200	6

			No activation	20
	3%	AAB	No activation	All strains (25)
Malvidine	6%	LAB*	87.5	1
			175	8
			350	4
			700	3
			No activation	22
	6%	*O. oeni*	43.8	1
			87.5	4
			175	8
			350	5
			No activation	9
	3%	AAB	No activation	All strains (25)

LAB*: Lactic acid bacteria except *O. oeni*.
Minimal activating concentration[1]: Minimal concentration that rendered 50% increase of bacterial growth. Growth activation for LAB* was determined by microtiter 12 h incubation with the activating agent and 6 % ethanol. Growth activation for *O. oeni* and AAB was determined by microtiter 24 h incubation with the activating agent and 6 % and 3 % ethanol respectively. The concentration range studied for L, M1 and M2 was 200 – 0.095 mg/l and for and malvidine 700 – 0.34 mg/l.

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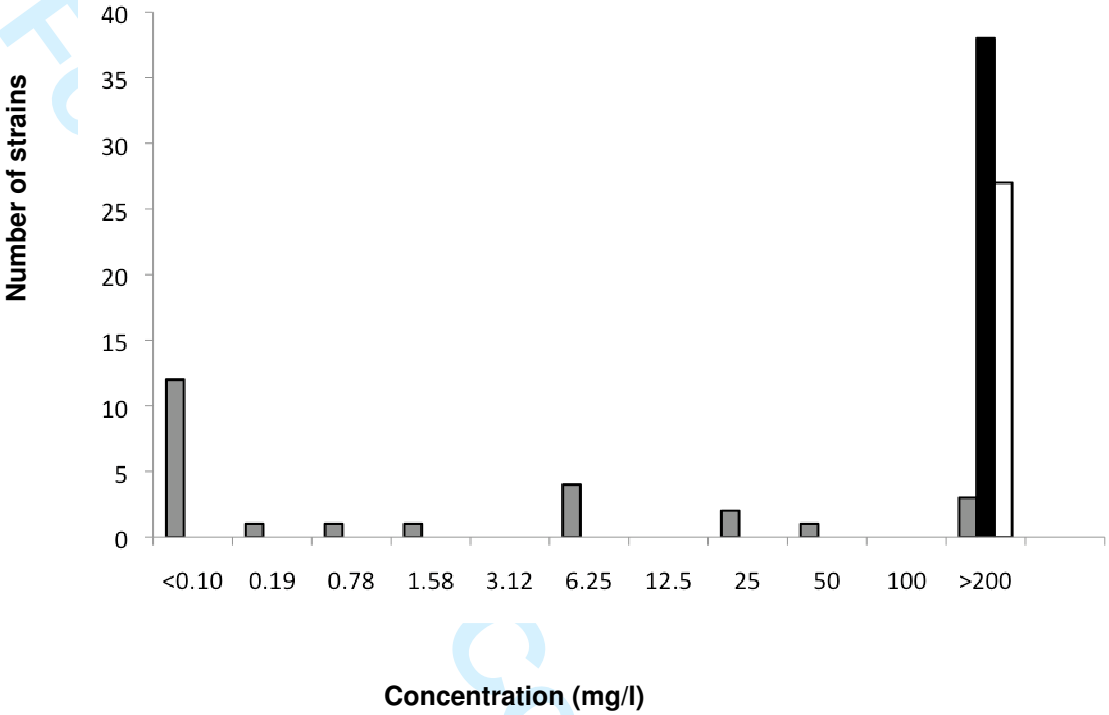


Figure 1. MIC values of the total polysaccharide extract from yeast lees (sample L) against ■ LAB*(lactic acid bacteria except *O. oeni*), □ *O. oeni* and ▨ AAB.

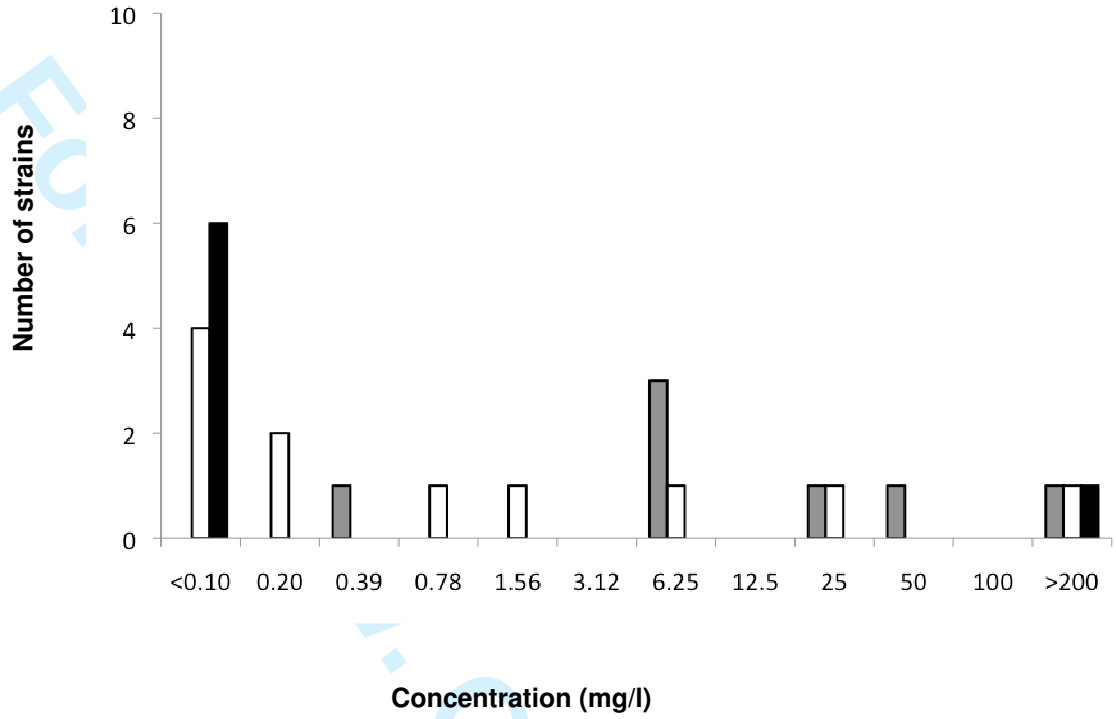


Figure 2. MIC values of the total polysaccharide extract from yeast lees (sample L) against ▒ *Gluconobacter*, □ *Acetobacter* and ■ *Gluconacetobacter* strains.

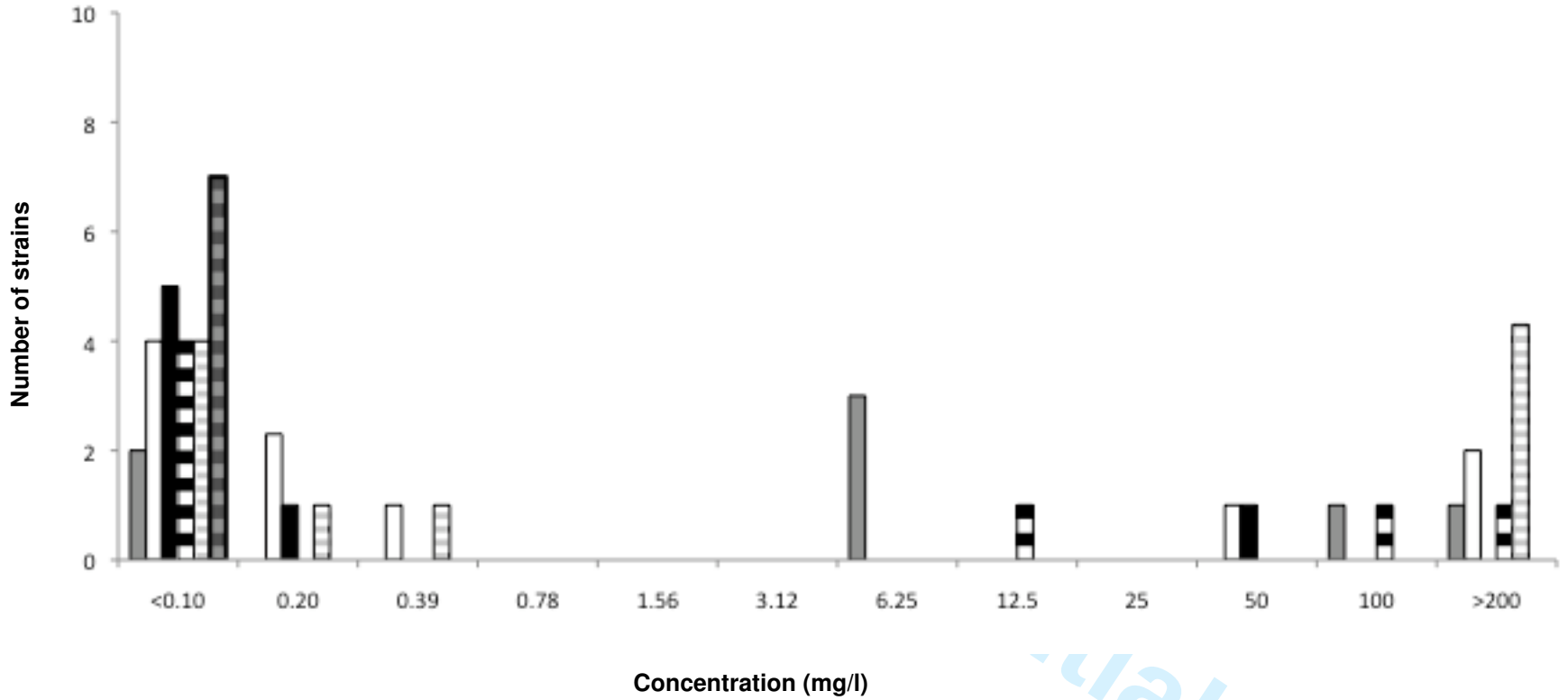
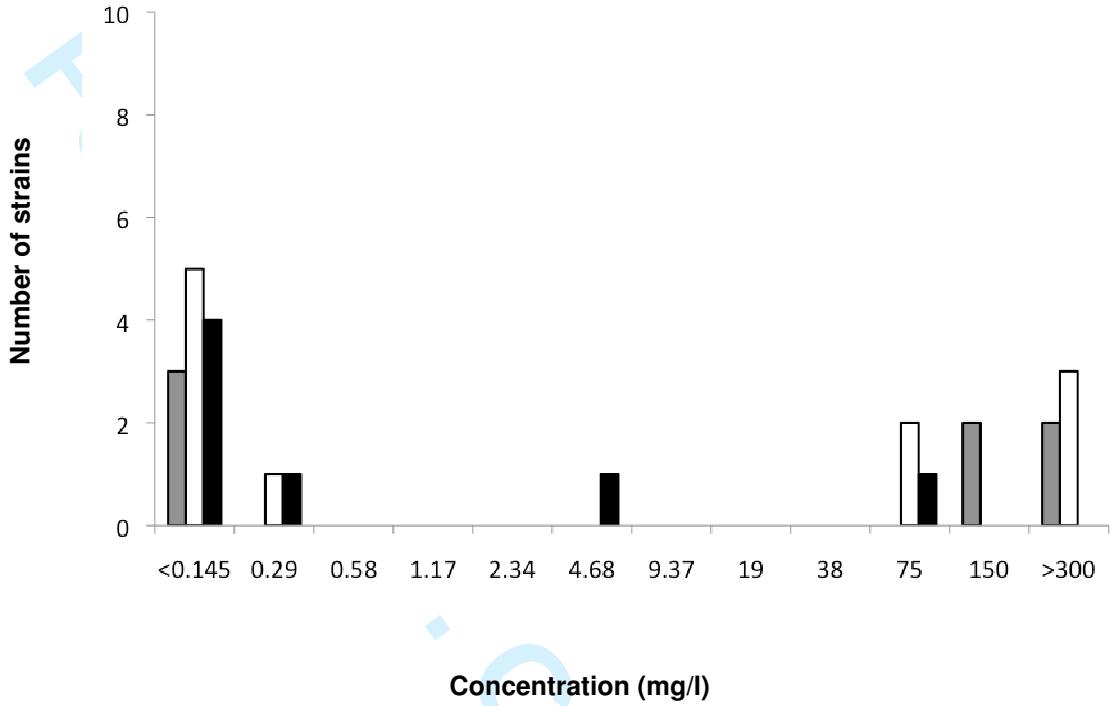


Figure 3. MIC values of intermediate (M1) and low (M2) molecular weight mannoproteins against *Gluconobacter, Acetobacter* and *Gluconacetobacter* strains.

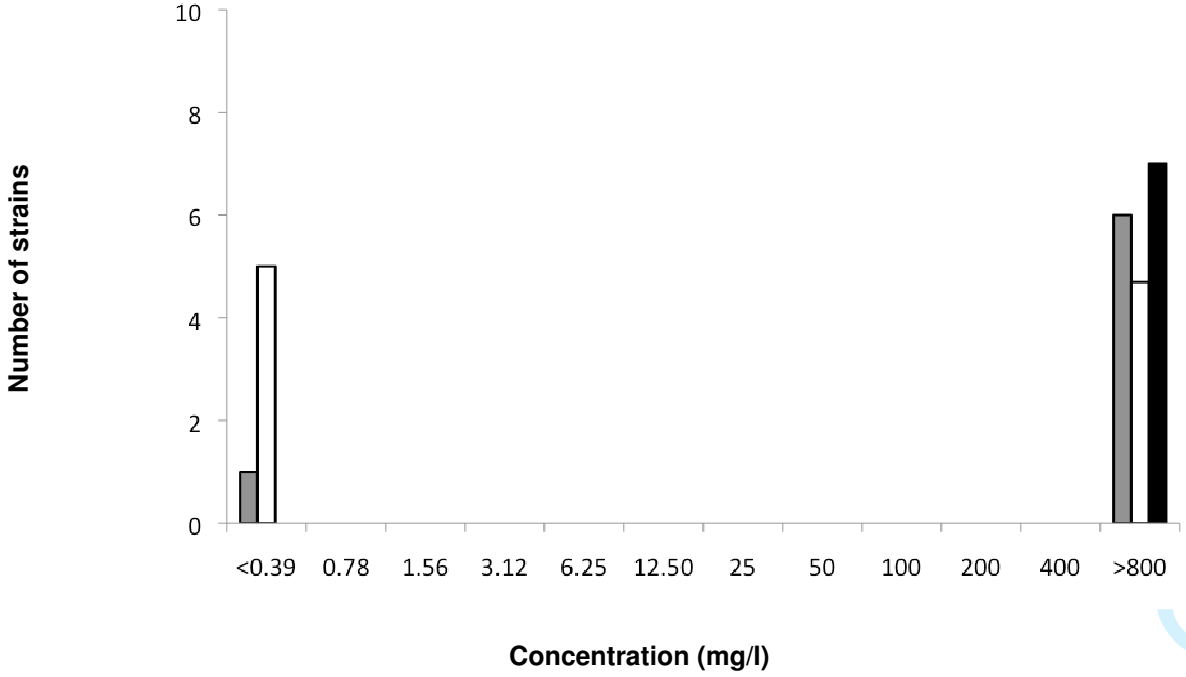
Intermediate molecular weight mannoproteins (M1) against: *Gluconobacter* *Acetobacter* *Gluconacetobacter*

Low molecular weight mannoproteins (M2) against: *Gluconobacter* *Acetobacter* *Gluconacetobacter*

A


Number of strains
10
8
6
4
2
0
<0.145
0.29
0.58
1.17
2.34
4.68
9.37
19
38
75
150
>300
Concentration (mg/l)

B


Number of strains
10
8
6
4
2
0
<0.39
0.78
1.56
3.12
6.25
12.50
25
50
100
200
400
>800
Concentration (mg/l)

C

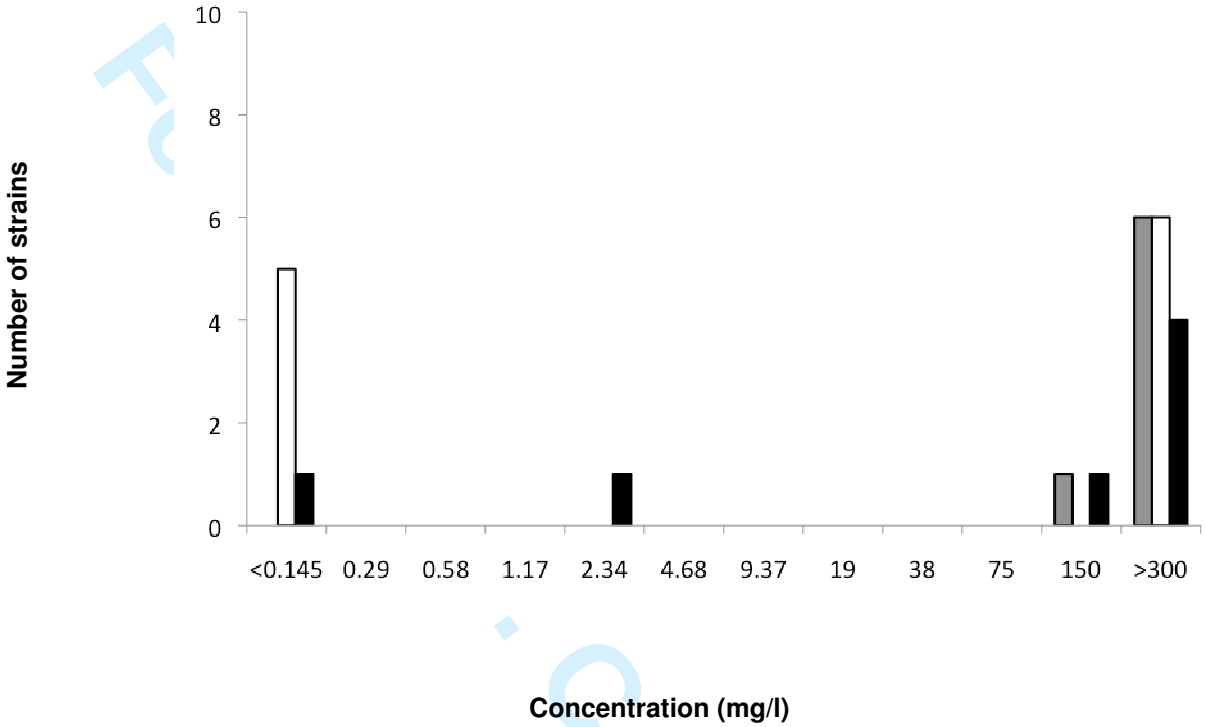


Figure 4: MIC values of polysaccharide extracts against:

□ *Gluconobacter,* □ *Acetobacter* and ■ *Gluconacetobacter* strains.

A: Grape must polysaccharide extract (sample G);

B: Wine polysaccharide extract (sample W)

C: Polysaccharide fractionation peak S1 (average molecular weight = 105 kD).

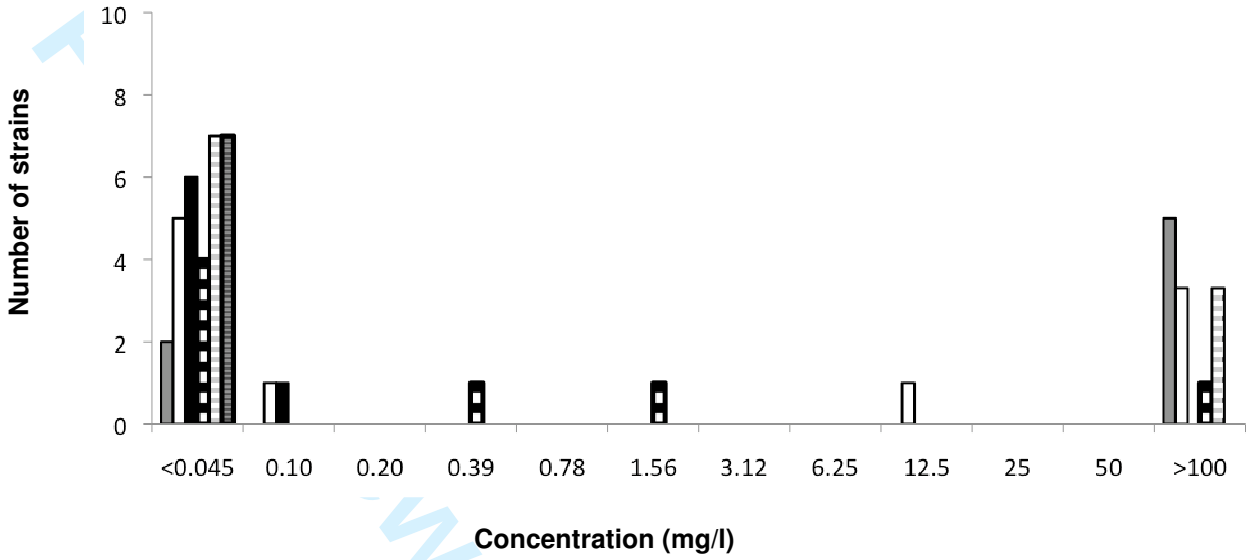


Figure 5: MIC values of intermediate (S2) and low (S3) molecular weight polysaccharide fractionation peaks (average molecular weight: 11.8 and 6 kD respectively) against *Gluconobacter, Acetobacter* and *Gluconacetobacter* strains.

S2 against: *Gluconobacter* *Acetobacter* *Gluconacetobacter*

S3 against: *Gluconobacter* *Acetobacter* *Gluconacetobacter*

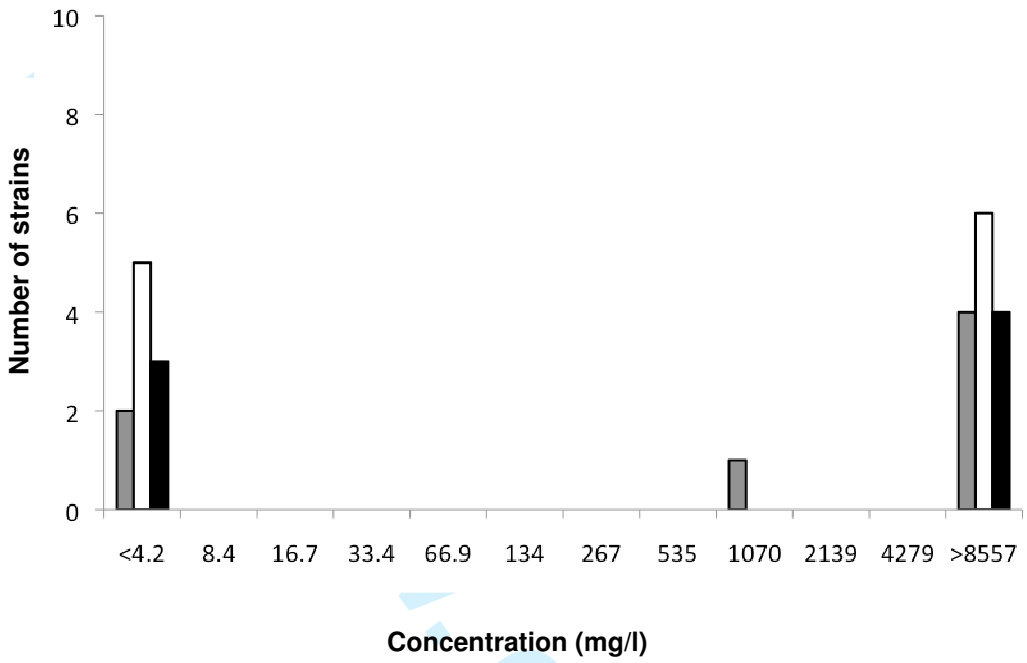


Figure 6: MIC values of catechin against : *Gluconobacter* *Acetobacter* *Gluconacetobacter*