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ORIGINAL RESEARCH PAPER

Characterization of the phenolic fraction from Argentine wine and its effect on viability and polysaccharide production of *Pediococcus pentosaceus*

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Abstract
Objectives To qualitatively and quantitatively characterize a low molecular weight phenolic fraction (LMF) of Malbec wine from Cafayate, Argentina, and evaluate its effect on viability and exopolysaccharide production of *Pediococcus pentosaceus* 12p, a wine spoilage bacterium.
Results The phenolic compounds detected were, in general, comparable to data previously reported but hydroxycinnamic acids were detected at higher concentrations than determined in other studies. Addition of LMF at identical concentrations present in wine or a four times concentrated LMF mixture to a synthetic wine-like medium produced a diminution in bacterial viability and exopolysaccharide production in the supernatant culture. Transmission electron microscopy revealed damage of bacterial cell integrity after 96 h of incubation only in the presence of four times concentrated LMF.
Conclusion This is the first time a low molecular weight phenolic fraction has been characterized in Cafayate wine and it has demonstrated a marked antimicrobial effect on an exopolysaccharide-producing wine spoilage bacterium.

Keywords Antimicrobial activity · Exopolysaccharide · Low molecular weight phenolic fraction · *Pediococcus pentosaceus* · Wine

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Introduction

During the winemaking process, certain lactic acid bacteria species belonging to the genus *Pediococcus*, could be related to deterioration of wine by exopolysaccharide (EPS) production (Manca de Nadra and Strasser de Saad 1995; Walling et al. 2005). In wineries, it is common practice to use SO_2 as an antimicrobial agent, but its overuse can generate negative effects on the wine organoleptic quality and lead to allergic disorders in susceptible consumers. Therefore, several countries have enforced restrictions regarding the maximum sulfur content in wines (Ribéreau-Gayon et al. 2006).

Nowadays, exploration of effective natural alternatives for the preservation of wine is a topic in order to replace or at least reduce the use of SO_2 during vinification (Guerrero and Cantos Villar 2015). Phenolic compounds are habitually present in plants and have recognized antimicrobial properties and beneficial effects on human health (Harborne and Williams 2000). A group of low molecular weight phenolic compounds, isolated from wine, have been described as having significant antibacterial activity against wine-spoilage lactic acid bacteria (García-Ruiz et al. 2009; Campos et al. 2009). The low molecular weight fraction of wines could exert an antibacterial effect against spoilage bacteria depending on the variety and concentration of phenolic compounds.

So far, the low molecular weight fraction of Malbec wine from Cafayate has not been characterized and it has never been assayed for its antimicrobial effect against wine spoilage bacteria. The aim of this study was to obtain qualitative and quantitative characterization of the phenolic compounds in the low molecular weight fraction isolated from a commercial Malbec wine produced in Cafayate, Salta, Argentina, and determine their antimicrobial effect in synthetic wine-like medium on the viability and exopolysaccharide production of *Pediococcus pentosaceus,* a wine spoilage bacterium.

Materials and methods

Wine samples

Samples were obtained from a commercial 2010 vintage varietal Malbec wine produced at 1700 m altitude in Cafayate in the Calchaquí Valleys in the northwest of Argentina. Samples were stored in the dark at 15–18 °C, and the bottles were opened immediately before assaying.

Isolation of the wine phenolic compound fraction

Liquid/liquid extraction was used to obtain a low molecular weight fraction of phenolic compounds (LMF). The extraction technique was performed with 750 ml wine according to Ghiselli et al. (1998) with slight modifications (Stivala et al. 2014). Ethanol was previously removed by vacuum distillation. An aliquot of 150 ml of the de-alcoholized wine was adjusted to pH 2 with 10 M HCl and extracted three times with 100 ml ethyl acetate. The organic fractions were pooled, dehydrated with 2.5 g anhydrous Na_2SO_4 and then evaporated to dryness under N_2 at 35 °C.

Chemical determinations

Total phenolic content of the LMF was determined using the Folin Ciocalteu microtechnique (Cicco et al. 2009). Briefly, the LMF was first dissolved in 40 % (v/v) methanol and then 100 µl correctly diluted samples were pipetted into separate test tubes, which were supplemented with 100 µl Folin–Ciocalteu reagent. After 2 min, 800 µl 5 % (w/v) Na_2CO_3 was added. The mixture was swirled and held at 40 °C for 20 min. The absorption of the blue solution was measured at 740 nm. A calibration curve of gallic acid was performed in order to express the phenolic content as gallic acid equivalent (mEq GAE).

Characterization of the polyphenolic fraction. HPLC analysis

The solid residue obtained after liquid/liquid extraction of the wine phenolic compound fraction was dissolved in 2 ml methanol/water (1:1, v/v), filtered through a 0.45 µm nylon membrane, and then injected into an HPLC with a reversed phase Nova-Pak C18 column (300 × 3.9 mm I.D., 4 µm; Waters Corp., Milford, MA) at 25 °C. Diode array detection was performed by scanning from 210 to 360 nm with an acquisition speed of 1 s. Identification of specific compounds was enabled by comparison of the spectra and retention times with standards. All individual phenolic compounds were confirmed by HPLC–DAD/ESI–MS (Fanzone et al. 2011).

Microorganism, culture medium and growth conditions

Pediococcus pentosaceus 12p was isolated from wine from Cafayate, Salta, Argentina (Strasser de Saad and Manca de Nadra 1987). MRS broth supplemented with tomato juice (15 % v/v) was used for microbial activation and the pH was adjusted to 4.8. After bacterial activation (36 h incubation), the *P. pentosaceus* 12p culture was collected and cells were washed three times with a sterile saline solution. The culture was subsequently inoculated in a synthetic wine-like medium (SWM). SWM was used in order to

mimic the wine composition and it sometimes facilitates maintenance of bacterial growth and/or viability. SWM contained a 1.7 % of YNB (Difco & BBLTM) solution, supplemented with (in g l^{-1}): glucose 5; fructose 3; L-malic acid 3; tartaric acid 4.0; K_2SO_4 0.1; $MgSO_4$ 0.025; $MnSO_4$ 1.0; adenine 0.05. The medium was also supplemented with 5 % (v/v) ethanol and the corresponding amino acids required for *P. pentosaceus* 12p growth (Aredes Fernandez et al. 2003). The pH was adjusted to 4.5 and the medium was sterilized by filtration through a 0.22 μm pore size nylon membrane.

Antibacterial activity assaying

Antibacterial assaying was performed by determination of growth parameters of the bacterium inoculated at 10^6 CFU ml^{-1} in SWM supplemented with the LMF obtained from Malbec wine. LMF was supplemented at the same concentration (1×) as determined in wine and at a four-times-increased concentration (4×), taking into account the total phenolic concentrations shown in Table 1 below. A control assay without addition of LMF was also carried out. The bacterium was incubated for 96 h at 28 °C under the different assay conditions. Immediately after bacterial inoculation and at the end of the incubation (96 h), bacterial viability was determined by counting viable cells on MRS-agar medium. The plates were incubated at 28 °C under microaerophilic conditions during 120 h. The results were used to determine the growth or death rate (K) and the change in the viable cells between the start of the inoculum and at the end of the incubation (A), as follows:

$$K[h^{-1}] = (\text{Ln } x/x_o)/(t \times \text{Ln } (2)) \quad (1)$$

$$A\ [\log\ \text{CFU ml}^{-1}] = \log\ (x - x_0) \quad (2)$$

where, x is the viable cell concentration at the end of the incubation time, x_0 is the viable cell concentration at the start of the inoculum.

Analysis of cell membrane damage

Immediately after inoculation and at the end of the incubation time, microbial cells were collected after centrifugation of SWM with or without addition of LMF at 10,000×g for 20 min at 4 °C. The pellets obtained under the different conditions (samples) were

Table 1 Composition of the low molecular weight fraction from Malbec wine (M) (mg l^{-1}) compared with concentrations in other wines

Concentration [mg l^{-1}]									
Compound	M	CS[a]	T[a]	M[b]	B[b]	CS[b]	Mr[b]	S[b]	Te[b]
Hydroxybenzoic acids/derivatives									
Gallic acid	22.7 ± 2.1	13.1 ± 1.4	19.9 ± 0.8	11.6 ± 1.3	20.7 ± 0.6	15.9 ± 1.0	15.1 ± 1.3	11.2 ± 1.2	11.1 ± 1.3
Protocatechuic acid	2.5 ± 0.2	1.9 ± 0.2	3.4 ± 0.4	1.4 ± 0.2	1.1 ± 0.1	1.2 ± 0.1	2.7 ± 0.2	1.5 ± 0.1	1.1 ± 0.1
Syringic acid	nd	nd	nd	3 ± 0.4	2.5 ± 0.3	1.6 ± 0.1	2.7 ± 0.2	3.1 ± 0.4	3.2 ± 0.2
Gentisic acid	nd	nd	nd	1.4 ± 0.3	1.5 ± 0.2	1.6 ± 0.1	1.3 ± 0.1	1.1 ± 0.2	1.8 ± 0.3
Methyl gallate	nd	3.1 ± 0.4	8.7 ± 0.6	2.3 ± 0.2	2.6 ± 0.2	2.5 ± 0.1	2.5 ± 0.3	1.6 ± 0.1	2.7 ± 0.2
Ethyl gallate	16.2 ± 1.5	16.4 ± 1.2	22.4 ± 2.1	7 ± 1.2	13 ± 0.8	8.8 ± 0.7	10.2 ± 0.8	4.7 ± 0.5	3.8 ± 0.6
Total	41.4 ± 4	34.5 ± 3.2	54.4 ± 3.7	26.7 ± 2.8	41.4 ± 0.8	31.6 ± 1.9	34.5 ± 2.6	23.2 ± 0.8	23.7 ± 1.3
Hydroxycinnamic acids/derivatives									
trans-Caftaric acid[c]	11.6 ± 1.2	7.1 ± 1.5	12.5 ± 2.3	5.6 ± 1.1	1.4 ± 0.1	3.6 ± 0.4	4.2 ± 0.5	5.4 ± 0.4	1.9 ± 0.3
cis-Caftaric acid[c]	3.8 ± 0.3	1.2 ± 0.1	0.6 ± 0.1	nd	nd	nd	nd	nd	nd
trans-Coutaric acid[c]	5.2 ± 0.5	5.8 ± 1.1	3.2 ± 0.4	3.9 ± 0.4	2 ± 0.1	2.3 ± 0.3	3.6 ± 0.4	6.1 ± 0.2	3.1 ± 0.2
cis-Coutaric acid[c]	6.1 ± 0.6	4.1 ± 0.7	7.3 ± 1.5	1.2 ± 0.2	0.6 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	2.5 ± 0.2	1.4 ± 0.3

Table 1 continued

Concentration [mg l^{-1}]									
Compound	M	CS[a]	T[a]	M[b]	B[b]	CS[b]	Mr[b]	S[b]	Te[b]
trans-Caffeic acid	*nd*	2.2 ± 0.04	1.8 ± 0.2	1.7 ± 0.2	5.6 ± 0.6	3.3 ± 0.5	3.3 ± 0.2	2.2 ± 0.1	1.8 ± 0.2
trans-Fertaric acid[c]	4.2 ± 0.4	2.3 ± 0.3	5 ± 0.4	3.55 ± 0.2	2.3 ± 0.04	2.73 ± 0.25	3.6 ± 0.15	2.5 ± 0.06	2.07 ± 0.03
trans-p-Coumaric acid	8.8 ± 0.9	4.4 ± 0.5	12.9 ± 0.9	2.6 ± 0.1	4.3 ± 0.6	3.3 ± 0.4	3.2 ± 0.2	2.1 ± 0.2	1.4 ± 0.4
Total	39.7 ± 0.3	27.1 ± 1.7	43.3 ± 1.8	18.6 ± 1.8	16.2 ± 1.1	16.5 ± 0.8	19.2 ± 0.8	20.8 ± 0.8	11.7 ± 0.4
Total phenolic acids	81.1	61.6	97.7	45.3	57.6	48.1	53.7	44	35.4
Alcohols/related compounds									
Tyrosol	12.7 ± 1.2	21.3 ± 4	15.2 ± 1.7	24.3 ± 4	24.9 ± 1.7	22.3 ± 2.1	30.1 ± 2.1	24 ± 1.7	20.9 ± 0.8
Tryptophol	nd	nd	nd	12.6 ± 2.3	10.2 ± 1.0	6.3 ± 0.5	9.5 ± 1.3	6.5 ± 0.9	14.4 ± 2.2
Total	12.7 ± 1.2	21.3 ± 4.0	15.2 ± 1.7	36.9 ± 6.2	35.1 ± 1.2	28.6 ± 2.5	39.6 ± 3	30.5 ± 2.6	35.3 ± 1.6
Stilbenes									
trans-Resveratrol-3-glucoside	1.9 ± 0.2	1.7 ± 0.1	3.4 ± 0.4	9.2 ± 1.0	3.2 ± 0.4	2.1 ± 0.3	4.1 ± 0.6	2.2 ± 0.4	1.9 ± 0.1
cis-Resveratrol-3-glucoside	nd	nd	nd	4.4 ± 0.7	2.9 ± 0.4	1.2 ± 0.2	4.8 ± 0.4	2.3 ± 0.1	2.7 ± 0.4
Total	1.9 ± 0.2	1.7 ± 0.1	3.4 ± 0.4	13.6 ± 1.7	6.1 ± 0.5	3.3 ± 0.5	8.9 ± 1	4.5 ± 0.4	4.6 ± 0.4
Total non-flavonoids	95.7 ± 8.7	84.6 ± 7.1	116.3 ± 8.2	95.8 ± 8.9	98.8 ± 1.9	80 ± 4.4	102.2 ± 6.3	79 ± 3.4	75.3 ± 2.5
Flavanols									
(+)-Catechin	19 ± 0.2	13.4 ± 3.6	12.9 ± 3.1	52.7 ± 6.5	58.7 ± 5.4	52.2 ± 3.7	44 ± 3.5	28.5 ± 5.7	19.9 ± 0.3
(−)-Epicatechin	13.9 ± 1.4	13.6 ± 0.8	13.3 ± 3.2	21.8 ± 3.3	34.8 ± 3.0	26 ± 1.4	31.7 ± 1.9	14.9 ± 3.6	12.3 ± 1.4
Procyanidin dimer 1	49.1 ± 4.7	40.5 ± 3.5	86.0 ± 4.4	16.0 ± 1.5	15.3 ± 1.9	10.7 ± 1.1	11.9 ± 1.2	7.9 ± 1.5	6.2 ± 0.6
Procyanidin dimer 2	nd	nd	nd	49.5 ± 1.1	58.4 ± 3.2	62.8 ± 3.2	44.6 ± 4.1	28.5 ± 3.8	36.0 ± 1.8
Procyanidin trimer 1	11.9 ± 1.6	9.6 ± 1.0	4.7 ± 0.5	15.6 ± 2.1	14.6 ± 0.4	9.9 ± 1	30.5 ± 2.6	3 ± 0.3	8.1 ± 0.4
Procyanidin trimer 2	5.9 ± 0.5	4.4 ± 0.5	7 ± 0.9	11.3 ± 1.2	14.2 ± 1.1	11.6 ± 1.2	15.2 ± 1.6	8.1 ± 1.0	8.5 ± 0.6
Procyanidin trimer 3	2 ± 0.1	8.5 ± 0.7	15.7 ± 1.1	12.9 ± 0.8	17.9 ± 1.7	12.2 ± 1	12.5 ± 0.9	12.6 ± 1.0	10.8 ± 0.2
Procyanidin trimer 4	6 ± 0.6	nd	nd	14.1 ± 2.8	7.0 ± 0.7	6.4 ± 0.6	nd	6.0 ± 0.6	4.7 ± 0.3
Total	107.8 ± 9.6	90 ± 4.4	139.6 ± 12.2	193.9 ± 19.1	220.9 ± 14.1	191.8 ± 10	190.4 ± 13.3	109.5 ± 17	106.5 ± 1.8
Flavonols									
Myricetin-3-glucuronide	nd	nd	nd	12.4 ± 1.9	9.3 ± 1.1	8.0 ± 1.1	8.8 ± 1.1	5.3 ± 0.7	6.6 ± 0.1
Myricetin-3-galactoside	nd	nd	nd	11.5 ± 1.4	15.8 ± 0.4	nd	nd	nd	nd
Myricetin-3-glucoside	nd	5 ± 0.5	3.1 ± 0.6	15.6 ± 2.7	nd	10.8 ± 1.1	19.9 ± 2.0	9.8 ± 1.2	18.7 ± 2.2
Quercetin-3-glucuronide	10.5 ± 1.1	10.4 ± 0.8	8.1 ± 0.7	11.9 ± 1.4	9.2 ± 0.9	6.8 ± 0.7	12.3 ± 1.8	9.6 ± 0.9	5.9 ± 0.7
Quercetin-3-galactoside	10.3 ± 0.9	16.2 ± 1.1	12.2 ± 0.4	nd	nd	nd	nd	nd	nd
Quercetin-3-glucoside	5.5 ± 0.5	5.7 ± 0.4	5.7 ± 0.4	9.4 ± 1.3	8.0 ± 1.1	8.5 ± 1.0	9.5 ± 1.4	11.7 ± 1.9	6.1 ± 1.1

Table 1 continued

Concentration [mg l^{-1}]									
Compound	M	CS[a]	T[a]	M[b]	B[b]	CS[b]	Mr[b]	S[b]	Te[b]
Quercetin-3-rhamnoside	4 ± 0.3	2.2 ± 0.4	3.7 ± 0.6	4.6 ± 0.6	4.5 ± 0.4	2.8 ± 0.2	5.6 ± 0.5	5.5 ± 0.4	3.2 ± 0.2
Isorhamnetin-3-galactoside	nd	8.6 ± .1.0	9.1 ± 1.3	7.4 ± 1.2	7.6 ± 0.9	11.3 ± 1.4	11.9 ± 1.2	nd	nd
Naringenin	7.8 ± 0.6	4.4 ± 0.4	10 ± 1.0	10.4 ± 1.4	5.3 ± 0.9	5.8 ± 0.4	8.1 ± 1	8 ± 0.4	5.7 ± 0.8
Syringetin-3-glucoside	9.1 ± 0.8	2.2 ± 0.2	3.3 ± 0.4	nd	3.1 ± 0.3	4.1 ± 0.6	nd	6.4 ± 0.9	4.3 ± 0.7
Kaempferol	nd	nd	nd	29.7 ± 5.6	19.9 ± 2.3	16.2 ± 1.3	21.7 ± 1.3	18.9 ± 1.4	24.9 ± 3.0
Total	47.2 ± 3.9	54.7 ± 3.2	55.2 ± 3.1	112.9 ± 8	82.7 ± 3.5	74.3 ± 5	97.8 ± 6.9	82.7 ± 3.7	80.8 ± 7.5
Dihydroflavonols									
Dihydroquercetin-3-glucoside	29.0 ± 2.8	nd	nd	55.4 ± 0.5	4.6 ± 0.3	7.6 ± 0.9	3.2 ± 0.3	2.6 ± 0.1	nd
Dihydroquercetin-3-rhamnoside	14.4 ± 1.3	nd	nd	4.7 ± 0.7	nd	nd	nd	nd	nd
Dihydrokaempferol-3-glucoside	20.5 ± 1.9	nd	nd	33.2 ± 1	10.4 ± 0.3	8.2 ± 0.3	9.5 ± 0.2	8.6 ± 0.2	nd
Dihydroflavonol unknown	2.1 ± 0.1	nd	nd	nd	nd	nd	nd	nd	nd
Total	66 ± 5.9	nd	nd	93.3 ± 2.0	15.0 ± 0.5	15.8 ± 0.9	12.7 ± 0.4	11.2 ± 0.2	nd
Total flavonoids	221 ± 21.5	144.7 ± 12	194.8 ± 13.3	400.1 ± 29.1	318.6 ± 16.5	281.9 ± 15.4	300.9 ± 20	203.4 ± 19.7	187.3 ± 8
Total phenolic compound	316.7 ± 28.9	229.3 ± 14.1	311.1 ± 17.0	495.9 ± 37.9	417.4 ± 17.4	361.9 ± 19.2	403.1 ± 25.7	282.4 ± 22.8	262.6 ± 6.8

Values are expressed as mean ± standard deviation. LMF of M (Malbec), CS (Cabernet Sauvignon), T (Tannat), B (Bonarda), Mr (Merlot), S (Shiraz) and Te (Tempranillo). Data from analytical determinations represent the average value of three determinations

nd not detected

[a] Stivala et al. 2014

[b] Fanzone et al. 2011

[c] Tartaric acid esters

examined by transmission electron microscopy. Samples were fixed in Karnovsky's solution (Karnovsky 1965) and incubated overnight at 4 °C. The fixed samples were washed three times with sodium phosphate buffer and then supplemented with 0.1 M sodium phosphate buffer/2 % (w/v) OsO_4 (1:1 v/v) and incubated for 12 h. Samples were then washed three times with distilled water and supplemented with phosphate buffer/uranyl acetate (2 g in 50 ml of distilled water) (v/v). After 30 min in the dark, the solution was discarded and the samples were washed with 70 % (v/v), 90 % (v/v) and 100 % ethanol, and subsequently dehydrated with 100 % ethanol and acetone. The bacterium was embedded in Spurr's resin and heated at 60 °C for 24 h. Ultrathin sections were obtained with an ultramicrotome, then mounted on copper grids and contrasted with uranyl acetate and lead citrate (Venable and Coggeshall 1965). The samples were observed with a transmission electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany).

Polysaccharide determination

After 0 and 96 h of incubation, cells were removed by centrifugation at 10,000×*g* for 20 min at 4 °C. Soluble polysaccharides present in the supernatant were precipitated with three volumes of absolute ethanol after overnight incubation at 4 °C. The solid residue was recovered by centrifugation at 14,000×*g* for 10 min at 4 °C, dried at 37 °C and finally dissolved in distilled water. The polysaccharide concentration of the samples was estimated using the phenol/sulfuric acid method with a glucose standard curve (Dubois et al. 1956).

Statistical analysis

The means and reproducibility of data were calculated based on three independent experiments performed in duplicate.

Results

Qualitative and quantitative analysis of the low molecular weight fraction. Characterization by HPLC–DAD

Table 1 shows the low molecular weight phenolic compounds identified and quantified in the LMF from Malbec wine. Phenolic compounds are grouped into non-flavonoids (hydroxybenzoic and hydroxycinnamic acids and their derivatives, stilbenes and phenolic alcohols) and flavonoids (flavanols, flavonols and dihydroflavonols). The total concentration of phenolic compounds of the LMF determined by HPLC was 316.7 mg l^{-1}, and this value is in the same order as that determined with the Folin Ciocalteu microtechnique (315.3 mg l^{-1} GAE). Regarding non-flavonoids, gallic acid was at the highest concentration of all hydroxybenzoic acids. Among the hydroxycinnamic acids, *trans-p*-coumaric acid was the only free acid detected at 8.8 mg l^{-1}. The remaining hydroxycinnamic acids were detected as tartaric acid esters, in accordance with Fanzone et al. (2011), with *trans*-caftaric acid at the highest concentration (11.6 mg l^{-1}).

The only stilbene compound detected was the glucoside of *trans*-resveratrol at 1.9 mg l^{-1}. Flavonoid compounds were the most abundant class of phenolic compounds detected, representing 69.8 % of the total phenolic compounds present in the LMF. Flavanols were the major kind of phenolic compounds detected (107.8 mg l^{-1}). The flavonols analyzed at the highest concentrations in the samples were quercetin-3-glucuronide and quercetin-3-galactoside, followed in descending order by syringetin-3-glucoside and naringenin. The most abundant dihydroflavonols detected were dihydroquercetin-3-glucoside and dihydrokaempferol-3-glucoside.

Effect of the phenolic fraction from Argentine red wine on growth and cell integrity of *Pediococcus pentosaceus*

Results in Table 2 show that the microorganism reached a viable cell count of 6.73 log CFU ml^{-1}

Table 2 Effect of the phenolic fraction (LMF) of Malbec wine on bacterial growth parameters

	P. pentosaceus 12p	
	K (h^{-1})	*A* (log CFU ml^{-1})
Control	0.02 ± 0.001	0.69 ± 0.06
LMF		
1×	−0.06 ± 0.005	−1.84 ± 0.16
4×	−0.17 ± 0.015	−4.86 ± 0.43

Values are expressed as mean ± standard deviation. Data from analytical determinations represent the average value of three determinations

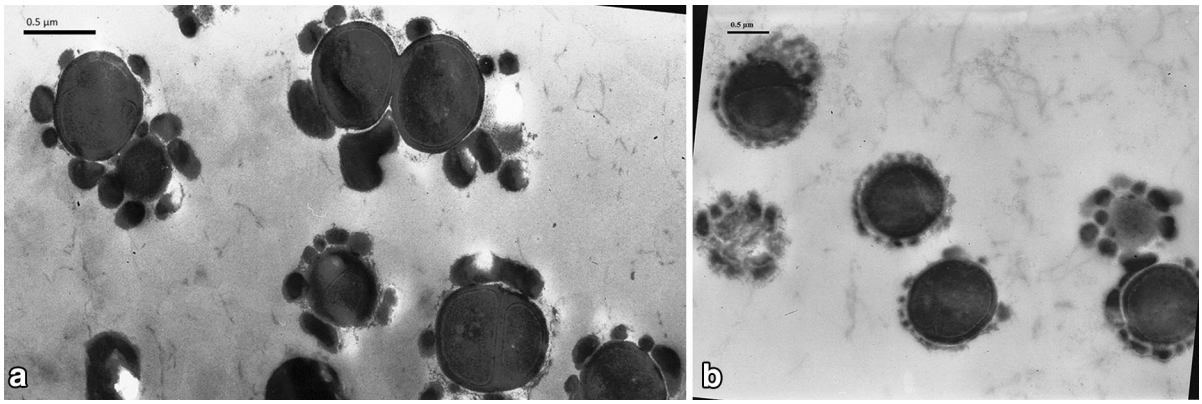

Fig. 1 Electron micrographs of ultrathin sections of *P. pentosaceus* 12p obtained after 96 h of incubation in SWM (**a**) and SWM supplemented with a four times concentrated fraction (4×) of phenolic compounds of low molecular weight from Malbec wine (**b**). *Marker bars* 0.5 µm

($A = 0.69 \log \text{CFU ml}^{-1}$; $K = 0.02\ \text{h}^{-1}$) after 96 h of incubation in SWM (control medium). Addition of LMF to SWM at the same concentration present in wine (1×) produced a drop in viable cell count after 96 h of incubation, determining an "*A*" value of $-1.84 \log \text{CFU ml}^{-1}$ and a *K* of $-0.060\ \text{h}^{-1}$. Addition of LMF at a concentration four times higher than normally in wine (4×) revealed a significant diminution in viable cell count ($A = -4.86 \log \text{CFU ml}^{-1}$) at a rate of $-0.17\ \text{h}^{-1}$ (*K*).

Changes in cell integrity of *P. pentosaceus* 12p cells after incubation in the presence of LMF were revealed by transmission electron microscopy. *P. pentosaceus* cell micrographs obtained from SWM after 96 h of incubation in the presence of 4 times concentrated LMF (4×) showed modifications in cell morphology with alterations in the microorganism cell integrity compared with SWM without addition of LMF (Fig. 1). Addition of LMF at a concentration normally observed in wine (1×) did not produce changes in cell morphology/integrity compared with control medium at the end of the incubation.

Effect of the phenolic fraction of Argentine wine on the polysaccharide production by *Pediococcus pentosaceus*

Table 3 shows that the EPS production of *P. pentosaceus* 12 p after 96 h of incubation in SWM control medium reached a value of 24.12 mg l^{-1}. Addition of SWM with LMF from Malbec at concentrations 1× and 4× produced a significant decrease in the EPS production of *P. pentosaceus* 12p with values close to zero.

Table 3 Determination of polysaccharides in the supernatant of a bacterial culture in SWM supplemented with LMF from Malbec wine

	Polysaccharides (mg l^{-1})
Control	24.1 ± 2.3
LMF	
1×	0.06 ± 0.01
4×	0.08 ± 0.01

Values are expressed as mean ± standard deviation. Data from analytical determinations represent the average value of three determinations

Discussion

This is the first time a low molecular weight fraction containing phenolic compounds is characterized in a Malbec wine varietal produced in Cafayate at an altitude of 1700 m in the Calchaquí Valleys in the northwest of Argentina. Additionally, the effect of the low molecular weight fraction was assayed on the growth and exopolysaccharide production of *Pediococcus pentosaceus* 12p, a lactic acid bacterium that can affect the wine organoleptic properties during winemaking.

The results obtained in the present study show concentrations of low molecular weight phenolic compounds comparable to those observed in previous

studies that used similar phenolic extraction procedures (Table 1).

The concentration of phenolic acids detected in LMF from Malbec wine (M) was 25 % higher than that reported for Cabernet Sauvignon and 20 % lower compared with Tannat, both from Cafayate (Stivala et al. 2014). Compared to wines from another region, the phenolic acid concentration of LMF of the Malbec (M) wine in this study was higher than that reported for several wine varietals from Mendoza, Argentina (Table 1). However, the phenolic acid concentration in wines from Italy is higher than that observed in the current study (La Torre et al. 2006; Ghiselli et al. 1998). Hydroxybenzoic acids/derivatives were the most abundant group in our samples and found at higher concentrations compared to Mendoza and other Cafayate wine varietals, except for T^1. The concentration of total hydroxycinnamic acid in LMF-M was nearly twice as high as in the LMF from Mendoza wines, except for CS^1 (1.5 times higher) and T^1 (10 % lower).

With respect to stilbenes, the only compound detected in LMF-M was the *trans*-isomer of resveratrol glucoside at 44 % lower than that reported for T^1 wine and at a concentration similar to CS^1. This compound was present at a lower concentration than in Mendoza wines (Table 1). However, the *trans*-resveratrol concentration in LMF-M was higher than in Italian wines (La Torre et al. 2006). Flavanols were the major kind of phenolic compounds present in LMF-M. Similar results have been reported for Mendoza (Table 1) and Italian wines (La Torre et al. 2006).

SWM supplemented with 1× LMF-M (same LMF composition as in wine) had a decreased bacterial viability with a marked loss of polysaccharide production. When LMF was concentrated four times (4×) and added to SWM, bacterial viability dropped dramatically, producing loss of viability at a higher death rate and with a significantly decrease in polysaccharides in the supernatant (Tables 2, 3).

The major antibacterial activity observed in wine is attributed to phenolic acids with hydroxycinnamic acids being the most effective in the control of lactic acid bacteria responsible for wine spoilage (Campos et al. 2009; García-Ruiz et al. 2009). García-Ruiz et al. (2009) demonstrated antimicrobial activity with 200 mg *p*-coumaric acid l^{-1} on *P. pentosaceus*. Campos et al. (2009) showed that phenolic acids, such as gallic acid and *p*-coumaric acid, at 2200 mg l^{-1} affected viability of wine spoilage bacteria such as *Lactobacillus hilgardii* and *Oenococcus oeni* by means of an increase in the cell membrane permeability. The concentrations mentioned are higher than those observed in the fraction characterized in the current study. García-Ruiz et al. (2011) determined that *trans*-resveratrol inhibited growth of *P. pentosaceus* at higher concentrations (IC_{50} = 715 - mg l^{-1}) in a complex culture medium supplemented with 6 % (v/v) ethanol. The flavanols (+)-catechin and (−)-epicatechin appear to show little inhibition of the growth of several strains of *L. hilgardii* and *P. pentosaceus* at 2000 mg l^{-1} (García-Ruiz et al. 2011).

A wine with polysaccharides at 95 mg l^{-1} is not considered ropy, opposed to a wine with polysaccharides at 300 mg l^{-1} (Ribéreau-Gayon et al. 2006). However, Lonvaud-Funel et al. (1993) considered that a polysaccharide production of approx. 100 mg l^{-1} is enough to give the wine an abnormal and unacceptable viscosity. Manca de Nadra and Strasser de Saad (1995) were the first to report production of exopolysaccharides by *P. pentosaceus* strains isolated from Argentine wines. The authors mentioned that *P. pentosaceus* strains 12p and E2p increased exopolysaccharide production in MRS culture medium in the presence of ethanol and SO_2.

To our knowledge, this is the first study that evaluates the effect of an extract of phenolic compounds obtained from wine on viability and exopolysaccharide production by a ropy *P. pentosaceus*, using a synthetic wine-like medium. The low molecular weight phenolic compounds of Malbec wine from Cafayate supplemented at a concentration determined in wine, was sufficient to decrease bacterial viability and markedly decrease polysaccharide production. Several studies have reported inhibitory activity against a number of LAB, including oenological strains of *Lactobacillus*, *Pediococcus* and *Oenococcus*, by phenolic extracts from different origin such as eucalyptus (García-Ruiz et al. 2012), different aromatic plants (García-Ruiz et al. 2012) and pure phenolic acids (Campos et al. 2009), but at significantly higher concentrations than those detected in wines; all these studies were performed in media that substantially differed from natural environment conditions.

On the basis of previous reports and the results obtained in the current study, it can be hypothesized that it is unlikely that a single phenolic compound at the concentrations found in wine could affect LAB growth. Instead, the complexity of the wine fraction

and the synergistic effect of certain components present in the fraction could explain the inhibitory activity of LMF-M on growth of *P. pentosaceus*. Consequently, the higher content of phenolic acids in Malbec wine from Cafayate could be positively related to the prevention of wine spoilage bacteria. The results obtained in the present study can be useful for the search of new natural alternatives to control detrimental lactic acid bacteria in wine, taking into account that phenolic compounds have certain advantages compared with potassium metabisulfite, which is habitually used. The use of sulfites is strictly controlled because high doses of them can cause organoleptic alterations in the final product (alteration of flavor through appearance of undesirable compounds such as mercaptans) and/or put health of consumers at risk causing allergic disorders (Ribéreau-Gayon et al. 2006). Other reports have stated that the use of sulfites at concentrations recommended in enology does not lead to lysis of the bacterial cell membrane but instead affects cell viability of *L. hilgardii* and *P. pentosaceus*. Indeed, cells entering in a viable but non-culturable state retained their metabolic activity and potential for wine spoilage (Millet and Lonvaud-Funel 2000; García-Ruiz et al. 2009). García-Ruiz et al. (2009) suggested that the phenolic compounds exhibit different antibacterial mechanisms compared with potassium metabisulfite, since they inactivate the bacteria which leads to cell death. Our results are in accordance with findings by these authors: the phenolic compound extract at high concentration (4X) was able to produce an alteration in the bacterial cell membrane with subsequent cell death. Studies are currently in progress to determine the most representative compound of the phenolic fraction and its role on the viability and metabolic behavior of several wine spoilage bacteria.

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