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EFFECT OF PHENOLIC COMPOUNDS FROM ARGENTINEAN RED WINES ON PATHOGENIC BACTERIA IN A MEAT MODEL SYSTEM

MARÍA J. RODRÍGUEZ VAQUERO[1,2,3], PEDRO A. AREDES FERNÁNDEZ[1], MARÍA C. MANCA DE NADRA[1,2] and ANA M. STRASSER DE SAAD[1]

[1]Reference Center for Lactobacilli (CERELA), Chacabuco, 4000, Tucumán, Argentina
[2]Faculty of Biochemistry, Chemistry and Pharmacy, National University of Tucumán, Ayacucho, 4000, Tucumán, Argentina

[3]Corresponding author. TEL: 54-381-4247752 ext 7065; FAX: 54-381-4247752; EMAIL: mariajo@fbqf.unt.edu.ar

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ABSTRACT

In this work, the effectiveness of phenolic compounds of different varieties of wines as antibacterial agent in a meat model system was determined. Total phenolic, flavonoid and flavanol compounds concentrations were greater in Merlot and Malbec wines compared with Cabernet Sauvignon variety. In meat, the best antibacterial effect of wine phenolic compounds against both bacteria was observed with Merlot and Malbec wine varieties at 4C, even when inhibitory effect was also observed at 20C. The lowest decimal reduction time was obtained with Merlot wine for *Listeria monocytogenes* and with Malbec and Merlot wines for *Escherichia coli*. From our results, we propose the use of wine phenolic compounds as natural biopreservatives for meat in combination with low temperatures. These natural products provide the additional human health benefit inherent to polyphenols properties.

PRACTICAL APPLICATIONS

The exploration of natural antimicrobials for food preservation receives increased attention due to a growing microbial resistance towards conventional preservatives, added to consumer awareness of natural food products. The antibacterial effect of individual phenolic compounds in culture media has been largely studied,, but there is no information about the antibacterial effect of natural combinations of polyphenols from different varieties of wines in meat. In this work, we demonstrate that phenolic compounds combination of Argentinean wines were effective in inhibiting the growth of two pathogenic bacteria that produce food contamination and illness in the consumer. Natural polyphenol combinations found in Argentinean wines could act as natural biopreservatives for meat in combination with low temperatures, achieving extension of the shelf life of food.

INTRODUCTION

Wine is a complex mixture of several hundred compounds present at different concentrations, some originating from the grapes and some metabolic by-products of yeast activity during fermentation (Soleas *et al.* 1997). The phenolic composition of wine is determined initially by the phenolic composition of the grapes used for making the wine (Ribéreau-Gayon *et al.* 1998) and exposure to sunlight and temperature are the main factors influencing the phenolic composition of grapes. The other factor that influences wine phenolic composition is the extraction of phenolic compounds from the grape skins and seeds during maceration (De Beer *et al.* 2003, 2005). Polyphenolic substances in wine are usually subdivided into three groups: nonflavonoids (e.g., gallic, protocatechuic, vanillic and caffeic acids), flavonoids (e.g., quercetin, rutin and catechin) and tannins (Singleton 1980). Phenolic compounds represent a common constituent of the human diet. They have a variety of beneficial effects on human health. Among these effects, these compounds have an important role in the antihypertensive and antithrombic effects (Jang *et al.* 1997). Epidemiological evidence indicates that the moderate consumption of wines reduces the incidence of coronary heart disease, atherosclerosis and platelet aggregation

(Tedesco *et al*. 2000). This greater protection may be due to the phenolic components of wines, which are particularly abundant in the red wine, since they behave as reactive oxygen species-scavengers and metal-chelators (Li *et al*. 2009). Some studies suggest that wine phenolic compounds also possess antimicrobial activity against various pathogens microorganisms in culture media (Weisse *et al*. 1995; Sugita-Konishi *et al*. 2001; Papadopoulou *et al*. 2005; Daglia *et al*. 2007; Rodriguez Vaquero *et al*. 2007a,b; Rodriguez Vaquero and Manca de Nadra 2008). Fresh meat and derivate products can be easily contaminated with microorganisms and, if not properly handled and preserved, support growth of spoilage and pathogen bacteria, leading to loss of quality and potential public health problems (Vernozy – Rozand *et al*. 2002). Refrigeration storage is usually the most common preservative method of fresh meat and meat products. In order to extend refrigerated storage time, antimicrobial and antioxidant additives especially of synthetic origin, are added to foods (Solomakos *et al*. 2008). The use of chemical additives is perceived by consumers as a health risk. Thus, the exploration of natural antimicrobials for food preservation receives increased attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives (Cushnie and Lamb 2005; Manca de Nadra and Rodríguez Vaquero 2009). The inhibitory effect of individual phenolic compounds on the growth of bacteria was demonstrated in culture media, but there is no information about the antibacterial effect of natural combinations of polyphenols from different varieties of wines in a meat model system, so in this point is focused this work.

As wine contains a complex mixture of phenolic compound that depends on the grape variety and winemaking process, the aim of this work was to determine the effectiveness of natural combinations of phenolic compounds of three Argentinean red wine varieties on *Listeria monocytogenes* and *Escherichia coli* viability in a bovine model meat.

MATERIALS AND METHODS

Strain Used and Preparation of the Inocula

The bacteria used as test organism were *L. monocytogenes*, isolated from human infection by public Hospital of Tucumán, Argentina and *E. coli* ATCC 35218 (American Type culture collection). *L. monocytogenes* was grown aerobically at 30C in brain heart infusion (BHI) broth (Britania, Argentina) medium, pH 7.0. *E. coli* was grown at 37C in nutrient broth and agar medium, pH 6.8. Before experimental use, cultures from solid medium were sub-cultured in liquid media, incubated for 24 h and used as the source of inocula for each experiment.

Enumeration Media

The selective medium used for enumeration of *L. monocytogenes* in meat was Palcam medium that contained (g/L): Agar base, 39.0, D-Glucose, 0.5, D-Mannitol, 10.0, esculine, 0.8, iron citrate and Ammonium, 0.5, phenol red, 0.08, lithium chloride, 15.0. The medium was supplemented with (UI/g): Polymyxin B, 50,000 UI, acriflavine HCL 0.0025 UI, ceftazidime, 0.01 UI. The medium used for enumeration of *E. coli* in meat was MacConkey medium (Britania, Agentine) that contained (g/L): peptone 17.0; plurypeptone 3.0; lactose 10.0; bile salts mixture 1.5; sodium chloride 5.0; neutro red 0.03; crystal violet 0.001 and agar 13.5.

Wine Samples

Three varieties of Argentinean red wines, Cabernet Sauvignon, Malbec and Merlot, were used. Wines were clarified by the addition of 30 mg/mL of activated charcoal, in order to eliminate phenolic compounds. All wine samples were filter-sterilized. The activated charcoal adsorbs phenolic groups on its surface, extracting only phenolic compounds present in wines, so they are used as control, without phenolic compounds. Wine samples were protected against sunlight and stored at 4C.

Analytical Determinations

Colorimetric Determination of Total Phenolic Compounds. Colorimetric determination of total phenolics was based on the procedure of Singleton and Rossi (1965). A standard curve of gallic acid was used. Results are expressed as milligram per liter gallic acid equivalents (GAE).

Nonflavonoid and Flavonoid Concentrations. 10.0 mL of the wine sample was mixed with 10.0 mL of diluted HCl (1:3) and 5.0 mL of an 8.0 mg/mL formaldehyde solution and incubated 24 h at room temperature in order to precipitate the flavonoid fraction (Zoecklein *et al*. 1990). The nonflavonoid phenol contents were determined in the filtrate using the procedure of Singleton and Rossi. The flavonoid content was obtained by the difference between total phenol and nonflavonoid content. All determinations were carried out in triplicate. Results are expressed as mg/L of gallic acid equivalents (GAE).

Flavonols Concentration. A standard curve of quercetin was used. 10.0 mg of quercetin was dissolved in ethanol 80% and diluted to 25, 50, 100, 150, 200, 250, 300 and 500 μg/mL. 0.5 mL of quercetin dilutions was mixed with 1.5 mL of ethanol 95%, 0.1 mL of $AlCl_3$ 10%, 0.1 mL of potassium

TABLE 1. TOTAL CONTENT, NONFLAVONOID, FLAVONOID AND FLAVONOL CONCENTRATIONS IN THREE WINES

		Grape variety		
Wines		Cabernet Sauvignon	Malbec	Merlot
Not clarified	Total*	2,300.01 ± 90.32	2,522.01 ± 80.67	2,704.02 ± 99.93
	Nonflanovoids*	690.82 ± 34.21	640.75 ± 32.11	659.21 ± 33.46
	Flavonoids*	1,609.21 ± 80.21	1,881.31 ± 94.34	2,044.82 ± 99.23
	Flavonols†	81.03 ± 6.22	168.34 ± 8.41	181.51 ± 9.12
Clarified	Total*	35.23 ± 1.82	25.11 ± 1.32	50.35 ± 2.92
	Nonflanovoids*	7.31 ± 0.36	6.13 ± 0.31	15.36 ± 0.77
	Flavonoids*	27.92 ± 1.45	19.04 ± 0.95	35.04 ± 1.73
	Flavonols†	5.61 ± 0.36	7.23 ± 0.46	8.14 ± 0.41

* mg/L GAE. Each value represents the average of three determinations ± SD.
† mg/L QE. Each value represents the average of three determinations ± SD.

acetate 1 mol/L and 2.8 mL of distilled water. The samples were incubated 30 min at room temperature. The absorbance was determined at 415 nm in spectrophotometer. Results are expressed as mg/L of quercetin equivalents (QE).

Antibacterial Activity of Polyphenols of Wine in a Meat Model System

Ten gram sample of lean cow meat was aseptically placed in stomacher bags. Ten milliliters of isotonic solution with Cabernet Sauvignon, Malbec and Merlot wine samples were added to the food to obtain a final concentration of 100 or 200 mg/L of polyphenols. The stomacher bags were inoculated at final concentration of 10^9 CFU/mL of *L. monocytogenes* or *E. coli* culture and were stomacher for 3 min to distribute the inocula. Stomacher bags were stored at 4C or 20C for 21 days. The survivors of *L. monocytogenes* or *E. coli* were enumerated at different time intervals 0, 4, 7, 14 and 21 days. The samples were serially diluted with isotonic solution and spread on Palcam agar or MacConkey agar. Plates were incubated for 24 h before enumeration. Controls were carried out for each wine, with the addition of the same volume of clarified wine (without phenolic compound). The effect of each wine on bacteria viability was compared with its corresponding clarified wine control. A second control without wine samples was carried out.

Decimal Reduction Time (D)

The time to reduce by 90% the viable cells of *L. monocytogenes* or *E. coli* was calculated graphically for each sample at 4C. Lower values were related with higher antibacterial activity.

Statistical Analysis

All experiments were carried out at least in triplicate. Experimental data were analyzed by ANOVA. Growth experimental data means were compared using Student's *t*-test.

RESULTS

Wine Composition

The phenolic compounds composition was determined for the three wine varietals used in this work. Total phenolic compounds, flavonoid, nonflavonoid and flavonol compounds concentrations are shown in Table 1. The concentration of total phenolic compounds in Merlot and Malbec wines were 15% and 9% higher than in Cabernet Sauvignon variety, respectively. Flavonoid compound concentrations were between 57% and 68% higher than nonflavonoid compound concentrations in all wines. Merlot wine contains in their composition 8% and 21.3% more flavonoid compounds than Malbec and Cabernet Sauvignon wines, respectively. The highest concentration of flavonol compounds was also found in Merlot wine being 7.3% and 55.2% lower in Malbec and Cabernet Sauvignon wines, respectively.

In order to eliminate the possible inhibitory effect of ethanol, its concentration was determined in all wines and clarified wines. Results in Table 2 show that all wine varieties and their clarified wines have the same ethanol concentration (12%), so clarified wines were used as ethanol control.

Clarified wines, in which between 93.1% and 99% of total phenolic compounds were removed, were used as controls. The only difference between wines and clarified wines was the concentration of phenolic compounds.

Antibacterial Effect

The effect of the different wines on the growth of the two species selected for this investigation was determined in a

TABLE 2. ETHANOL CONCENTRATIONS IN THREE WINES

	Grape variety		
	Cabernet Sauvignon	Malbec	Merlot
Wines	Percentage of ethanol (%)		
Not clarified	12 ± 1	12 ± 1	12 ± 1
Clarified	12 ± 1	12 ± 1	12 ± 1

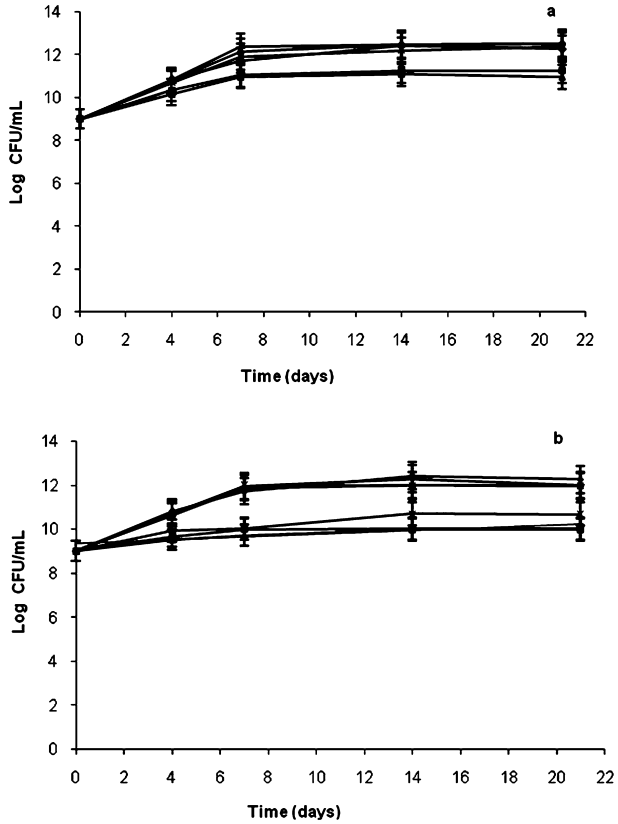

FIG. 1. SURVEY OF *L. MONOCYTOGENES* IN A MEAT MODEL SYSTEM SUPPLEMENTED WITH WINES STORAGE AT 20C: (a) 100 mg/L AND (b) 200 mg/L
(◆) Control, wines: (▲) Merlot (■) Malbec and (X) Cabernet Sauvignon. Clarified wines: (●) Merlot (☒) Malbec and (+) Cabernet Sauvignon. Each point represents the average value of three determinations.

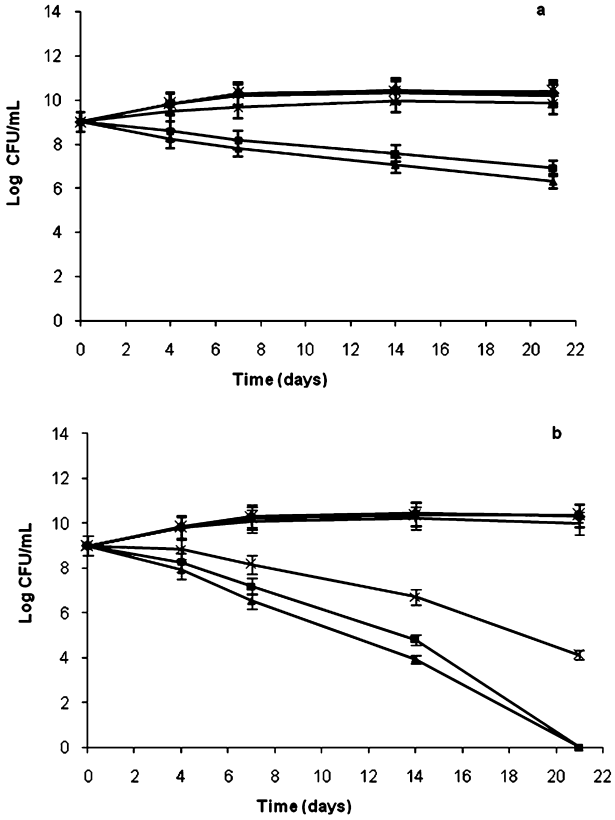

FIG. 2. SURVEY OF *L. MONOCYTOGENES* IN A MEAT MODEL SYSTEM SUPPLEMENTED WITH WINES AT 4C: (a) 100 mg/L AND (b) 200 mg/L
(◆) Control, wines: (▲) Merlot (■) Malbec and (X) Cabernet Sauvignon. Clarified wines: (●) Merlot (☒) Malbec and (+) Cabernet Sauvignon. Each point represents the average value of three determinations.

meat model system, supplemented individually with 100 and 200 mg/L of polyphenols from the three wine varieties, at 20C and 4C.

Figure 1 show the results for *L. monocytogenes* at 20C. In a control meat model system, without wine samples addition, *L. monocytogenes* population increased in 3.26 logarithmic cycles at 21 days. Bacterial growth was not modified by the addition of clarified wines. The addition of 100 mg/L of polyphenols from Cabernet Sauvignon, Malbec or Merlot wines (Fig. 1a), decreased 26.4%, 33.4% and 44.1% the *L. monocytogenes* growth with respect to control meat at 21 days. A diminished of 44%, 65.6% and 66.9%, on the growth of the bacteria was observed with the addition of 200 mg/L of total polyphenols from Cabernet Sauvignon, Malbec and Merlot wines, respectively, at 21 days (Fig. 1b).

At 4C (Fig. 2), in a control meat model system, *L. monocytogenes* growth 1.3 log cycles at 21 days and no significant differences were observed with the individual addition of clarified wines. Treatment with 100 mg/L of polyphenols of Malbec and Merlot wines produced a reduction of the number of inoculated cells of 2.1 and 2.7 log cycles at 21 days, respectively (Fig. 2a). In the same conditions, Cabernet Sauvignon wine diminished the bacterial growth 28% with respect to control. At 21 days of incubation, no viable cells of *L. monocytogenes* were detected with the addition of 200 mg/L of polyphenols of Malbec and Merlot wines. A decrease of inoculated cells by 4.9 log cycles was observed with the addition of 200 mg/L of polyphenols of Cabernet Sauvignon wine (Fig. 2b).

The effect of wine polyphenols of the three wine varieties on the growth of *E. coli* in a meat model system at 20C is shown in Fig. 3. In a control meat model system incubated at 20C, the microorganism growth 3.64 log cycles at 21 days. The growth of *E. coli* with the individual addition of clarified wines was similar to the meat control. When 100 mg/L of total polyphenols from Cabernet Sauvignon, Malbec or Merlot wines was individually added to meat (Fig. 3a), *E. coli* growth decreased for accounting of 32.7%, 65.9% and 32.6%, respectively, with respect to their controls at the end of incubation.

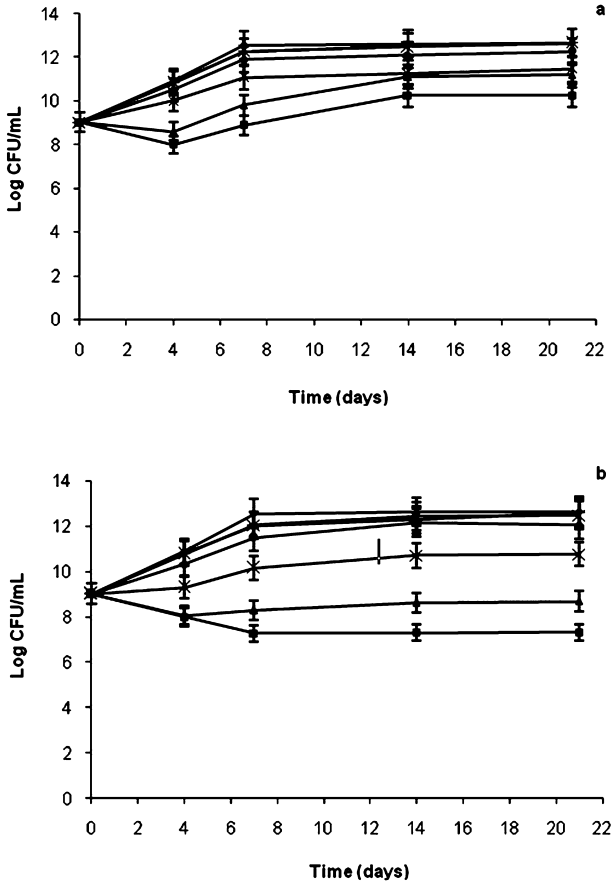

FIG. 3. SURVEY OF *E. COLI* IN A MEAT MODEL SYSTEM SUPPLEMENTED WITH WINES STORAGE AT 20C: (a) 100 mg/L AND (b) 200 mg/L
(◆) Control, wines: (▲) Merlot (■) Malbec and (X) Cabernet Sauvignon. Clarified wines: (●) Merlot (Ж) Malbec and (+) Cabernet Sauvignon. Each point represents the average value of three determinations.

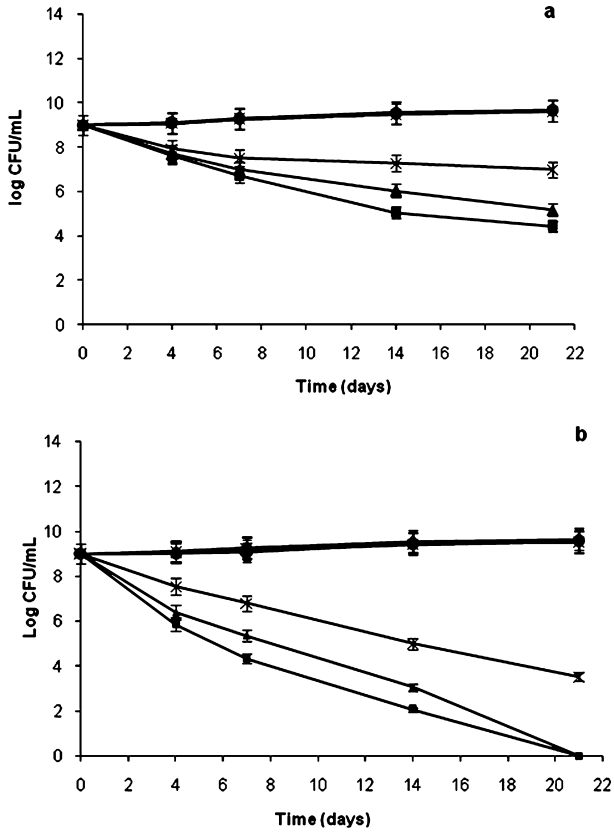

FIG. 4. SURVEY OF *E. COLI* IN A MEAT MODEL SYSTEM SUPPLEMENTED WITH WINES AT 4C: (a) 100 mg/L AND (b) 200 mg/L
(◆) Control, wines: (▲) Merlot (■) Malbec and (X) Cabernet Sauvignon. Clarified wines: (●) Merlot (Ж) Malbec and (+) Cabernet Sauvignon. Each point represents the average value of three determinations.

The addition of 200 mg/L of total polyphenols from Cabernet Sauvignon (Fig. 3b) diminished 50.8% the growth of *E. coli* as regard to the control. Phenolic compounds of Malbec and Merlot wines produced a reduction of the number of inoculated cells by 1.69 and 0.31 log cycles, respectively.

At 4C (Fig. 4), *E. coli* growth 0.63 log cycles at 21 days of incubation. Treatment with 100 mg/L of Cabernet Sauvignon, Malbec and Merlot wines reduce the number of inoculated cells by 2.0, 4.57 and 3.82 log cycles at 21 days, respectively (Fig. 4a). With 200 mg/L of polyphenols of Malbec and Merlot wines, no viable cells of *E. coli* were detected at the end of incubation (Fig. 4b). A decreased of inoculated cells by 5.49 log cycles was observed with the addition of 200 mg/L of polyphenols of Cabernet Sauvignon wine.

Table 3 shows the *D* values of *L. monocytogenes* and *E. coli* in presence of total polyphenols of wines in a meat model system, calculated from Figs 2b and 4b, respectively. The lowest decimal reduction times of *L. monocytogenes* were observed with 200 mg/L of Merlot wine (2.4 days). The *D* values of polyphenols of Malbec and Cabernet Sauvignon wines were 2.0- and 3.2-fold higher than the *D* value of Merlot wine, respectively. The lowest decimal reduction times of *E. coli* were observed with Malbec (1.3 days) and Merlot wine (1.5 days).

TABLE 3. DECIMAL REDUCTION TIMES OF *L. MONOCYTOGENES* AND *E. COLI* CALCULATED GRAPHICALLY FOR EACH WINE SAMPLE AT 4C

	Decimal reduction time (days)			
	L. monocytogenes		*E. coli*	
Wine samples	100 mg/L	200 mg/L	100 mg/L	200 mg/L
Cabernet Sauvignon	–	7.70	3.7	2.70
Malbec	8.90	4.70	2.8	1.3
Merlot	3.50	2.40	3.0	1.5

DISCUSSION

The wine varieties used in this investigation are traditionally produced in Argentine and consumed widely around the world. In the present study, we investigated the relation between the phenolic compound content in each wine variety and their antibacterial effect against *L. monocytogenes* and *E. coli*, bacteria frequently detected in meat, with economic impact in the food industry. The results showed that total phenolic compound concentration was greater in Merlot and Malbec wines compared with Cabernet Sauvignon variety. In the three wine varieties, flavonoid fraction was greater than nonflavonoid fraction. Merlot and Malbec wine varieties exhibit a higher concentration of flavonoid and flavanol compounds than Cabernet Sauvignon wine variety.

Taking into account that: (1) the bacterial growth was the same in a meat model system without wines (control) and in meat added with clarified wines and (2) there were no differences in the ethanol concentrations between wines and clarified wines, the inhibitory effect observed in a meat model system added with wines was not due to ethanol concentration.

It is well known the synergistic effect of preservative compounds with low temperatures but the experiences were also carried out at 20C in the aim to know the effect of wine phenolic compounds in the situations where the cold chain is interrupted. In meat, the best antibacterial effect of wine phenolic compounds against *L. monocytogenes* and *E. coli* was observed with Merlot and Malbec wine varieties. As expected, even when at 20C there was an important inhibitory effect, the phenolic compounds of wines were more effective at 4C, producing reduction of the number of inoculated cells at the two concentrations assayed, as evidenced by the values of decimal reduction times. Our results are in agreement with several works that demonstrated that the use of low temperatures enhanced the inhibitory ability of phenolic compounds (Bahk *et al*. 1990; Beuchat *et al*. 1994). Ultee *et al*. (1999) demonstrate that phenolic compounds are more effective at 4C than at 20C, and its mode of action depends on migration into bacterial membranes, which are less fluid at chill temperatures. The differences observed in the antibacterial effect between the wine varieties could be related to the differences in phenolic compounds concentration but also with the phenolic compounds profile in each wine.

Before this work, there is no information about the antibacterial effect of natural combinations of polyphenols from different varieties of Argentinean wines in a meat model system.

The best antibacterial effect of phenolic compounds combinations of Merlot or Malbec wines is probably due to the higher flavonol concentration than in natural combination of polyphenols of Cabernet Sauvignon wine.

CONCLUSION

According to these experiences, we can propose the use of polyphenol combination found in wines or grapes, rich in flavonol compounds, as sources of phenolic compounds useful as natural biopreservatives for meat in combination with low temperatures. These natural products, as well as extend the shelf life of food, provide the additional human health benefit inherent to polyphenols properties.

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