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Phenolic Compounds from Wine as Natural Preservatives of Fish Meat

María José Rodríguez-Vaquero[1,2]*, *Pedro Aredes Aredes-Fernández*[2] *and María Cristina Manca de Nadra*[1,2†]

[1]Faculty of Biochemistry, Chemistry and Pharmacy, National University of Tucumán, Ayacucho 471, 4000 Tucumán, Argentina

[2]Reference Center for Lactobacilli (CERELA), Chacabuco 145, 4000 Tucumán, Argentina

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Summary

The aim of this work is to investigate the antibacterial effect of phenolic compound combinations and total polyphenols of Argentinean red wine varieties against *Escherichia coli* ATCC 35218 and *Listeria monocytogenes* using commercial fish meat as model food. Rutin-quercetin combination and three wine varieties (Cabernet Sauvignon, Malbec and Merlot) caused cellular death of both bacteria on fish meat at 4 °C. Rutin-quercetin combination was effective on fish meat even at 20 °C. Clarified wines did not affect the bacteria, indicating that wine polyphenols are responsible for the observed effect. The use of wine phenolic compounds as antibacterial agent could be used to prevent contamination and extend the shelf life of fish meat. A big finding of this work is the use of rutin–quercetin combination as preservative for the conservation of fish meat and its transport to the fish market, which is an effective antibacterial agent even when the transport temperature is not constant.

Key words: phenolic compounds, *L. monocytogenes*, *E. coli*, fish meat, antibacterial effect

Introduction

Food safety is of fundamental concern to both consumers and food industry, especially as the number of reported cases of food-associated infections continues to increase. Microorganisms are the major cause of contamination and spoilage of fish meat, producing dangerous products and changes in the sensory properties, rendering it unsuitable for human consumption.

Listeria monocytogenes is a Gram-positive bacterium responsible for the severe foodborne illness, listeriosis. Several reports associate listeriosis with the consumption of contaminated seafood (*1*). Although most healthy humans are not significantly affected by low doses of the bacteria, the pathogen can be more potent for people with weak immune systems or during pregnancy (*2*,*3*). Among severe infections, listeriosis has been associated with a mortality rate as high as 30–40 % (*3*). Furthermore, this microorganism cannot survive cooking temperature but is capable of growing at refrigeration temperature (*4*).

Escherichia coli is a Gram-negative bacterium, the primary pathogen on meat products (*5*). Some strains of *E. coli* can cause diarrhoea, urinary tract infections, inflammation and peritonitis in immunosuppressed patients such as children and elderly people (*6*,*7*). As a consequence, the absence of *E. coli* from foods can be used to assess its sanitary quality (*8*).

Understanding the growth of contaminant microorganisms in seafood and other foods is crucial for the development of preservation techniques and subsequent reduction of losses due to contamination and spoilage. There is a constant striving to produce safer food and to develop new antimicrobial agents. Concerns over the

*Corresponding author; Phone: ++54 381 424 7752 ext. 7067; Fax: ++54 381 424 8169; E-mail: mariajo@fbqf.unt.edu.ar

safety of some chemical preservatives and negative consumers´ reaction towards preservatives they perceive as chemical and artificial have prompted an increased interest in developing more natural alternatives. Hence, recently there has been interest in testing natural products, including plant-derived compounds, for antilisterial properties as these may be used as natural preservatives in foods (*9*). Phenolic compounds represent a common constituent of the human diet; they are found in fruit, vegetables and flowers as well as tea and wine (*10*). They have a variety of beneficial effects on human health, including anti-inflammatory, anti-allergic, antioxidant and cytotoxic activities (*11*). Phenolic compounds are subdivided into three groups: phenolic acids (*e.g.* gallic, protocatechuic, vanillic and caffeic acids), flavonoids (*e.g.* quercetin, rutin and catechin) and tannins (*12*). Wine is a complex mixture of several hundred compounds present at different concentrations. The major ones are water, ethanol, glycerol, sugars, organic acids and salts, while aliphatic and aromatic alcohols, amino acids and phenolic compounds are present at much lower concentrations. The phenolic composition of wine is determined by the phenolic composition of the grapes used for making the wine (*13*), and exposure to sunlight and temperature are the main factors influencing the phenolic composition of grapes.

Several investigators demonstrated that wines possess antibacterial activity (*14–17*), but the exact mechanisms responsible for it are not fully understood (*18*). Different components of wine have been proposed to contribute to its antimicrobial activity, some authors give emphasis to the role of wine phenolics and others accentuate the role of non-phenolic constituents of wine, such us organic acids, ethanol, *etc.* (*19*).

We had previously found that *Proteus mirabilis*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Listeria monocytogenes* exhibited different sensitivities towards different concentrations of phenolic compounds and wines in standard laboratory media (*14–16*). Later we demonstrated that the use of wine phenolic compounds as natural biopreservatives for bovine meat was effective in reducing the viability of *E. coli* and *L. monocytogenes* in a food system model (*20*).

At present, there are no reports regarding the antibacterial effect of phenolic compounds on fish meat model in the scientific literature, most reports are about trials conducted in laboratory media, and consequently little is understood about their effectiveness when applied to fish meat.

The aim of this work is to investigate the antibacterial efficiency of three phenolic compound combinations and total phenolic compounds of three Argentinean red wine varieties on *E. coli* and *L. monocytogenes* viability in a fish meat model system at 4 and 20 °C.

Materials and Methods

Strains used and preparation of the inocula

The bacteria used as test organisms were *Listeria monocytogenes*, isolated from human infection in a public hospital in Tucumán, Argentina, and *Escherichia coli* ATCC 35218 (American Type Culture Collection (ATCC; Manassas, VA, USA). *L. monocytogenes* was grown aerobically at 30 °C in brain heart infusion (BHI) broth (Britania, Buenos Aires, Argentina), pH=7.0. *E. coli* was grown at 37 °C in nutrient medium, pH=6.8. Before experimental use, cultures from solid medium were subcultured 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 *Listeria monocytogenes* in meat was PALCAM (Britania) medium that contained (in g/L): agar base 39.0, D-glucose 0.5, D-mannitol 10.0, esculine 0.8, ferric ammonium citrate 0.5, Phenol Red 0.08 and lithium chloride 15.0. The medium was supplemented with (UI/g): polymyxin B 50 000, acriflavine hydrochoride 0.0025 and ceftazidime 0.01. The medium used for enumeration of *E. coli* in meat was MacConkey medium (Britania) that contained (in g/L): peptone 17.0, plurypeptone 3.0, lactose 10.0, bile salts mixture 1.5, sodium chloride 5.0, Neutral Red 0.03, Crystal Violet 0.001 and agar 13.5.

Samples

Pure phenolic compounds

Gallic acid was obtained from Merck (Darmstadt, Germany), protocatechuic acid, caffeic acid, quercetin and rutin were purchased from ICN Pharmaceuticals (Bryan, OH, USA). The purity of all phenolic compounds was >98 %. All phenolic compounds were dissolved in ethanol 99.8 % (Merck) and filter-sterilized through a 0.22-μm membrane filter (Durapore PVDF, Millipore, Billerica, MA, USA). The selected combinations of the used phenolic compounds were: gallic and protocatechuic acids, gallic and caffeic acids, and quercetin and rutin. These combinations were selected on the basis of previous results in culture medium (*21,22*).

Wines

Three varieties of Argentinean red wines, Cabernet Sauvignon, Malbec and Merlot, were used. Clarified wines were used as controls, without phenolic compounds. Clarification was done by the addition of 30 mg/mL of activated charcoal, in order to eliminate phenolic compounds. All wine samples were filter-sterilized. Wine samples were protected against sunlight and stored at 4 °C. The total phenolic compounds, phenolic acids, flavonoid and flavonol concentrations of the three wines used were determined in a previous work (*14,20*).

Antibacterial activity on fish meat model system

Effect of the combinations of pure phenolic compounds

Lean fish meat, obtained from a local market was stored at –20 °C. A mass of 10 g of meat was aseptically placed in stomacher bags and 10 mL of isotonic solution with combinations of phenolic compounds were added to obtain a final concentration of 100 or 200 mg/L in a ratio of 1:1. The selected combinations of compounds for this experiment were: gallic and protocatechuic acids (G-P), gallic and caffeic acids (G-C), and quercetin and

rutin (Q-R). The stomacher bags were inoculated with 10^9 CFU/mL of *E. coli* and were homogenized for 3 min. The bags were stored at 4 or 20 °C for 21 days. The control was the inoculated fish meat in a stomacher bag containing 10 mL of isotonic solution with ethanol 5 %.

Effect of wine polyphenols

Samples of lean fish meat (10 g) were aseptically placed in stomacher bags. Isotonic solution (10 mL) with Cabernet Sauvignon, Malbec and Merlot wine samples was added to the meat to obtain final concentrations of 100 or 200 mg/L of total polyphenols. The stomacher bags were inoculated at a final concentration of 10^9 CFU/mL of *L. monocytogenes* or *E. coli* culture and were stomached for 3 min to distribute the inocula. Then they were stored at 4 or 20 °C for 21 days. The survivors of *L. monocytogenes* or *E. coli* were enumerated at different time intervals: 0, 4, 7, 14 and 21 day. The samples were serially diluted with isotonic solution and spread on PALCAM 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) instead of wine. The effect of each wine on the viability of bacteria was compared with its corresponding clarified wine control. A second control was carried out without wine samples.

Decimal reduction time

The time to reduce the viable cells of *L. monocytogenes* or *E. coli* by 90 % was calculated graphically for each sample at 4 °C.

Statistical analysis

All experiments were carried out at least in triplicate. Experimental data were analyzed by ANOVA. Mean values of the growth experimental data were compared using Student's *t*-test.

Results

Survey of E. coli and L. monocytogenes in fish meat with the addition of phenolic compound combinations

In control fish meat, without phenolic compounds, the growth of inoculated cells of *E. coli* increased by 3.98 logarithmic cycles on day 21 of incubation at 20 °C. Table 1 shows the reduction in the number of viable cells of *E. coli* in fish meat with the combinations of phenolic compounds in 21 days of storage at 20 and 4 °C.

At 20 °C, the addition of 100 mg/L of G-P, G-C or R-Q combinations decreased the growth of *E. coli* by 51.3, 68.3 and 100 %, respectively, with respect to control meat. With 200 mg/L of G-P or G-C combinations, the inhibitory effect on the growth increased by 64.8 and 92.2 %, respectively. R-Q combination was the only one that caused the death of 90 % of the inoculated cells, in 14 days of storage.

At 4 °C, in control fish meat *E. coli* growth increased by 0.08 log cycles after 21 days of incubation. All phenolic compound combinations at the concentration of 100 mg/L caused the death of the bacterium, with G-C and Q-R combinations being the most effective, and the lowest decimal reduction time in days (*D*) value (1.9 days) was found with R-Q combination. After 21 days, with 200 mg/L of G-P or G-C, no viable cells were detected; the same effect was observed with R-Q combination at 14 days, with the lowest *D* value.

In a control fish meat model, without phenolic compounds added, the number of *L. monocytogenes* cells increased by 3.87 log cycles in 21 days of incubation at 20 °C. At 4 °C the number of viable cells of *L. monocytogenes* increased by 0.60 log cycles at the end of incubation. Table 2 shows the reduction in the number of viable cells of *L. monocytogenes* in fish meat with the combinations of phenolic compounds in 21 days of storage and at 20 and 4 °C. At 20 °C, 100 mg/L of G-P, G-C and Q-R inhibited the growth of microorganisms (35.9, 75.5 and 90.7 %, respectively), without causing cellular death. All combinations at the concentration of 200 mg/L caused cellular death; Q-R combination was the most effective, with a *D* value 3- and 1.2-fold lower than the *D* values obtained with G-P and G-C, respectively. At 4 °C, all combinations at the concentration of 100 mg/L caused cellular death, with G-C and Q-R showing the lowest *D* values. The addition of 200 mg/L of phenolic compounds intensified the cellular death, and in 14 days no bacteria were detected in fish meat containing G-C or Q-R combination.

Table 1. Reduction of viable cell number of *E. coli* in fish meat with added combinations of phenolic compounds in 21 days of storage at 20 and 4 °C

Combination of phenolic compounds	Log cycle reduction of *E. coli*											
	t/°C											
	20						4					
	γ(TP)/(mg/L)											
	100			200			100			200		
	C	I	*D*	C	I	*D*	C	I	*D*	C	I	*D*
G-P	2.04	–	–	2.58	–	–	5.0	4.92	4.3	9.08	9.0	2.0
G-C	2.73	–	–	3.37	–	–	9.08	9.0	2.1	9.08*	9.0*	1.6
Q-R	4.04	0.05	–	5.51	1.53	13.9	9.08	9.0	1.9	9.08*	9.0*	1.5

TP=total polyphenols, C=log cycle reduction with respect to control, I=log cycle reduction with respect to inocula, *D*=decimal reduction time in days; –=no inhibition observed. *At day 14 no viable cells were detected

Table 2. Reduction of viable cell number of *L. monocytogenes* in fish meat with added combinations of phenolic compounds in 21 days of storage at 20 and 4 °C

Combination of phenolic compounds	Log cycle reduction of *L. monocytogenes*											
	t/°C											
	20						4					
	γ(TP)/(mg/L)											
	100			200			100			200		
	C	I	*D*	C	I	*D*	C	I	*D*	C	I	*D*
G-P	1.39	–	–	4.87	1.0	21.0	2.6	2.0	9.0	3.96	3.36	4.4
G-C	2.92	–	–	5.83	1.96	8.5	4.65	4.05	5.2	9.6*	9.0*	1.2
Q-R	3.51	–	–	6.67	2.8	6.9	6.15	5.55	4.0	9.6*	9.0*	1.3

TP=total polyphenols, C=log cycle reduction with respect to control, I=log cycle reduction with respect to inocula, *D*=decimal reduction time in days; –=no inhibition observed. *At day 14 no viable cells were detected

E. coli and L. monocytogenes in fish meat containing three wine varieties

Fig. 1 shows the growth of *E. coli* in fish meat supplemented individually with the three wine varieties, at 20 °C. In control meat sample the number of *E. coli* cells increased by 3.98 log cycles in 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 the *E. coli* growth by 37.8, 75.9 and 52.03 %, respectively, with respect to the control in 21 days. A decrease of 46.4 % of the growth of bacteria was observed with the addition of 200 mg/L of total polyphenols from Cabernet Sauvignon wine compared to the control (Fig. 1b). Polyphenols from Malbec and Merlot wines caused the death of the inoculated cells, Malbec being more effective, with lower *D* value (Table 3).

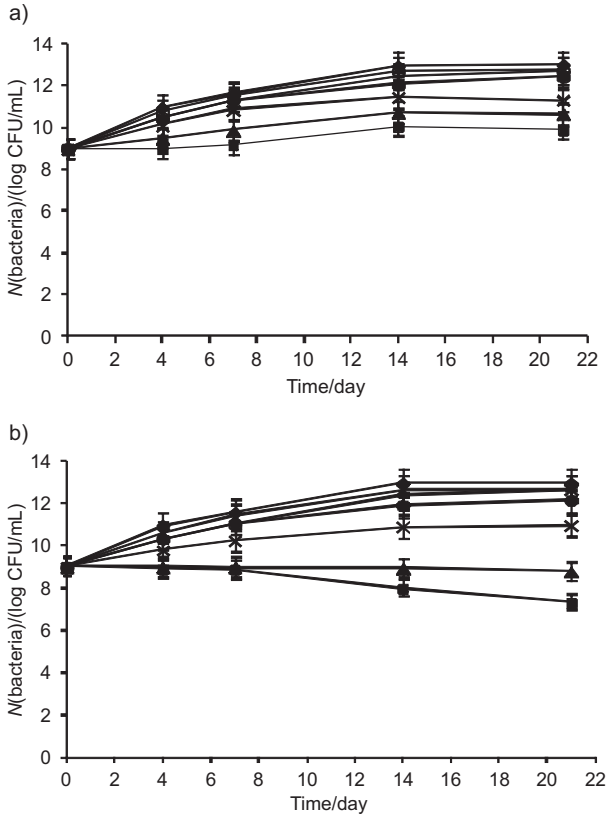

Fig. 1. Survey of *E. coli* in fish meat supplemented with wines stored at 20 °C: a) 100 and b) 200 mg/L. (◆) Control, wines: (▲) Merlot (■) Malbec and (×) Cabernet Sauvignon. Clarified wines: (●) Merlot, (○) Malbec and (×) Cabernet Sauvignon. Each point represents the average value of three determinations

Table 3. Decimal reduction time (*D*) of *E. coli* calculated graphically for each wine sample at 4 °C

Wine sample	*D*(*E. coli*)/day	
	γ(TP)/(mg/L)	
	100	200
Cabernet Sauvignon	7.20	3.10
Malbec	2.60	1.90
Merlot	3.80	2.40

TP=total polyphenols

At 4 °C (Fig. 2), in control *E. coli* increased by 0.07 log cycles in 21 days. Both treatments, with the addition of 100 or 200 mg/L of polyphenols from the three wine varieties caused the cellular death; Malbec polyphenols were the most effective with the lowest *D* value (Table 3).

Fig. 3 shows the growth of *L. monocytogenes* in control fish meat sample and in the fish meat supplemented with the three wine varieties at 20 °C. The growth rate of *L. monocytogenes* in the control meat (with clarified wines) was similar to the control without wines. In the control meat sample, the microorganism growth was 3.87 log cycles in 21 days of incubation. With 200 mg/L of total polyphenols from Cabernet Sauvignon added to the meat, *L. monocytogenes* growth decreased by 73.7 % (Fig. 3b). The same concentrations of polyphenols from Malbec or Merlot wines caused the death of inoculated cells, Merlot being the most effective, with the lowest *D* value (Table 4).

The growth of *L. monocytogenes* increased by 0.60 log cycles in 21 days at 4 °C (Fig. 4). Treatment with 100 or 200 mg/L of polyphenols from Cabernet Sauvignon, Mal-

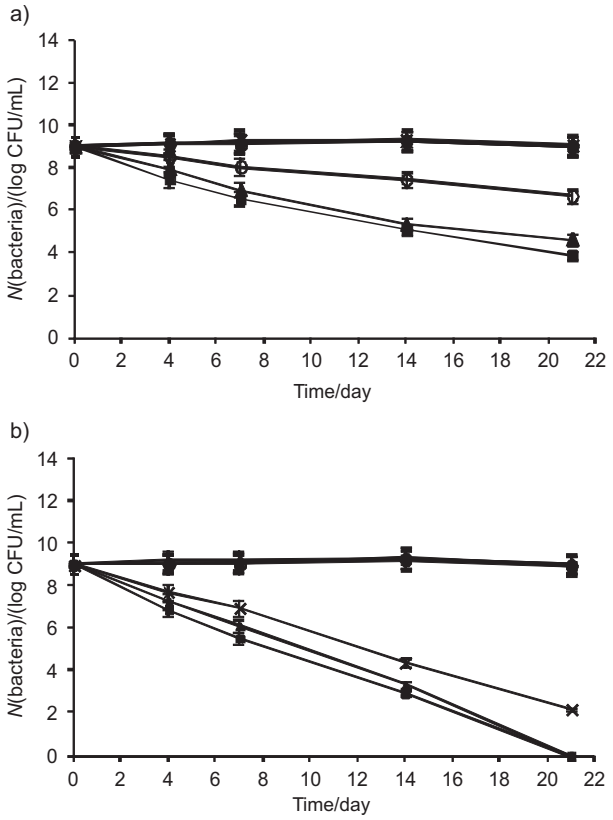

Fig. 2. Survey of *E. coli* in fish meat supplemented with wines stored at 4 °C: a) 100 and b) 200 mg/L. (◆) Control, wines: (▲) Merlot (■) Malbec and (×) Cabernet Sauvignon. Clarified wines: (●) Merlot, (○) Malbec and (×) Cabernet Sauvignon. Each point represents the average value of three determinations

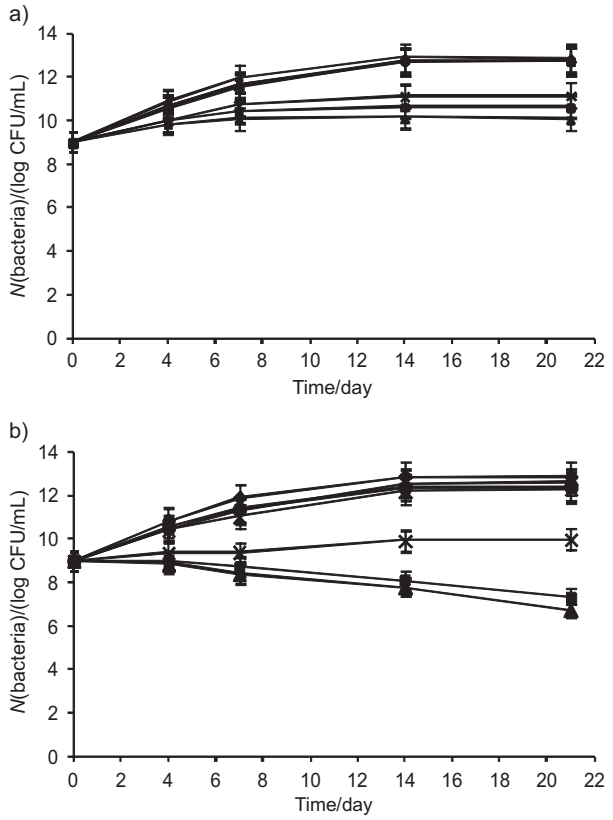

Fig. 3. Survey of *L. monocytogenes* in fish meat supplemented with wines stored at 20 °C: a) 100 and b) 200 mg/L. (◆) Control, wines: (▲) Merlot (■) Malbec and (×) Cabernet Sauvignon. Clarified wines: (●) Merlot, (○) Malbec and (×) Cabernet Sauvignon. Each point represents the average value of three determinations

bec and Merlot caused cellular death. With 200 mg/L of polyphenols from Merlot or Malbec, no viable cells were detected at 14 and 21 days, respectively. The lowest *D* value (1.3 days) was observed with polyphenols from Merlot wines (Table 4).

Discussion

In this study the antibacterial activity of the combinations of phenolic compounds and total polyphenols of three red wine varieties on a fish meat model system was investigated against *E. coli* and *L. monocytogenes*, bacteria frequently detected in meat, with economic impact on the food industry. The wines used in this investigation were produced traditionally in Argentina and are consumed widely around the world. Also, the influence of temperature on the antibacterial activity as well as the relationship among the phenolic compounds in each wine variety were investigated.

As expected, even though there was an important inhibitory effect at 20 °C, the combinations of phenolic compounds were more effective at 4 °C, causing cellular death at the two concentrations assayed, with the lowest value of decimal reduction time for rutin-quercetin combination. This combination at 200 mg/L was also effective at 20 °C and caused cellular death of both bacteria, which is a great discovery since it could prevent contamination of fish meat with these pathogenic bacteria,

Table 4. Decimal reduction time (*D*) of *L. monocytogenes* calculated graphically for each wine sample at 4 °C

Wine sample	*D*(*L. monocytogenes*)/day	
	γ(TP)/(mg/L)	
	100	200
Cabernet Sauvignon	13.40	6.00
Malbec	6.00	2.00
Merlot	3.70	1.00

TP=total polyphenols

without the need of using low temperature. This is an important finding for the transport of fish meat, during which the cold chain can be disrupted at various times, and for the conservation during the storage of this meat.

With respect to the antibacterial effect of wine polyphenols, the best effect against *L. monocytogenes* and *E. coli* viability in fish meat was observed when using polyphenols from Merlot and Malbec wine varieties at 4 °C. The differences observed in the antibacterial effect could be related to the differences in phenolic compound concentrations and composition among the studied wine varieties. In previous work, Rodríguez Vaquero *et al*. (*14, 20*) reported that total phenolic, flavonoid and flavanol concentrations were greater in Merlot and Malbec wines

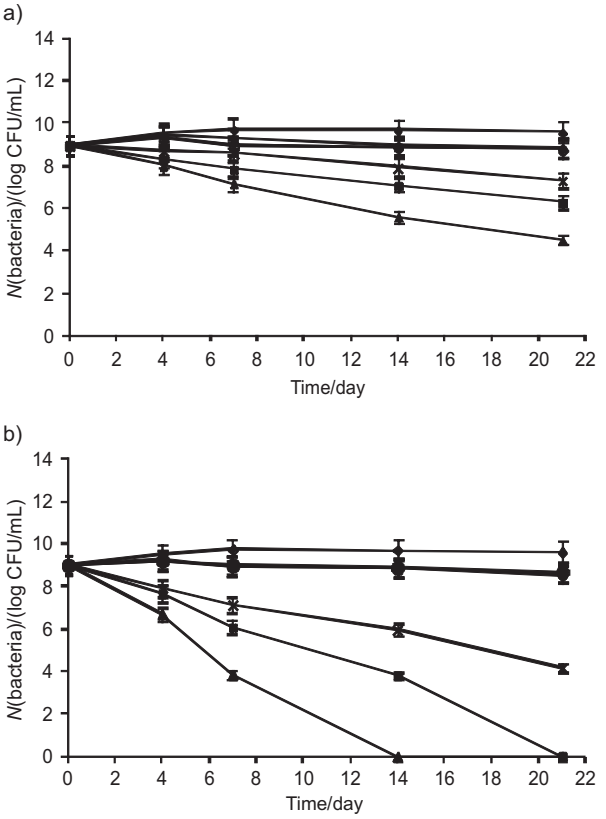

Fig. 4. Survey of *L. monocytogenes* in fish meat supplemented with wines stored at 4 °C: a) 100 and b) 200 mg/L. (◆) Control, wines: (▲) Merlot (■) Malbec and (×) Cabernet Sauvignon. Clarified wines: (●) Merlot, (○) Malbec and (×) Cabernet Sauvignon. Each point represents the average value of three determinations

compared to Cabernet Sauvignon. Besides, Rodríguez Vaquero *et al*. (*15,16*) reported that flavonol compounds, such as rutin and quercetin, had the best antibacterial activity in a culture medium. Merlot and Malbec wines had higher concentrations of flavonol compounds than Cabernet Sauvignon, which could be related to the major antibacterial activity observed in these wines.

The clarification was effective in removing phenolic compounds of the three wines. There were no significant differences in ethanol concentration or pH between wines and clarified wines, so clarified wines were added to control meat and they were inactive against both bacteria, indicating that the phenolic compounds present in wines were responsible for the antibacterial effects.

Papadopoulou *et al.* (*23*) indicate that some wine phenolic acids are probably the most active components in inhibiting the growth of Gram-positive and Gram--negative bacteria and yeasts. Boban *et al.* (*18*) observed that the antibacterial activity of wines could not be related to their total phenolic and resveratrol content, antioxidant capacity, ethanol content, or pH. They indicate that the antimicrobial activity of complex solutions such as intact wine cannot be exclusively attributed to its phenolic or non-phenolic constituents.

Other authors (*24,25*) reported that low temperatures enhanced the inhibitory activity of phenolic compounds. Refrigeration at or below 4 °C in combination with other preservation factors (*e.g*. modified atmosphere packaging) is already used widely for extending the shelf life of many food products. In this work, phenolic compounds are more effective at 4 than at 20 °C, and their mode of action depends on their migration into bacterial membranes (*26*), which are less fluid at lower temperatures.

A group of ten colleagues determined that sensorial changes in fish meat were not significant at the concentrations of phenolic compounds used in this study. To corroborate these results, studies of sensorial evaluation are carried out by professional and qualified panellists.

Conclusion

The use of wine phenolic compounds as antibacterial agents at refrigerator temperature could be a good method to prevent fish meat contamination and to extend the shelf life of the product. Besides, phenolic compounds could provide additional benefits inherent to their natural biological properties and health benefits. Futhermore, the use of rutin and quercetin combination as a preservative compound has been found excellent for the conservation of fish meat as a safe product and its transport to the fish market.

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