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Influence of phenolic compounds from wines on the growth of *Listeria monocytogenes*

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Abstract

The anti-microbial properties against *Listeria monocytogenes* of pure flavonoids rutin, catechin and quercetin; non-flavonoids gallic, vanillic, protocatechuic and caffeic acids and total polyphenols of three Argentinean wines, Cabernet Sauvignon, Malbec and Merlot varieties were investigated. The non-flavonoid caffeic acid and the flavonoids rutin and quercetin were the compounds with higher inhibitory activities on *L. monocytogenes* growth. The knowledge of the anti-listerial effect of different wines varieties could be the basis to demonstrate if the wine consumption with a meal may collaborate in the health protection against some foodborne organisms such as *L. monocytogenes*.

Keywords: Phenolic compounds; Anti-microbial activity; Wine; Growth inhibition; *Listeria monocytogenes*

1. Introduction

Wine composition depends on the grapes used to make the wine and on the vinification conditions (Cheynier, Hidalgo Arellano, Bouquet, & Mountounet, 1997). Phenolic compounds are responsible for some of the major organoleptic properties of wines, in particular color and astringency. Polyphenolic substances in wine are usually subdivided into two groups: flavonoids and non-flavonoids. The non-flavonoids in wine, phenols with only one aromatic ring, are derivatives of hydroxycinnamic acid and of hydroxybenzoic acid. The flavonoids have a common core, the flavane nucleus, consisting of two benzene rings (A and B) linked by an oxygen-containing pyrane ring (Van de Wiel, van Golde, & Hart, 2001).

Interest in phenolic compounds of grapes and wines has increased in recent years because of their potential beneficial effects on human health (Caillet, Salmiéri, & Lacroix, 2006; Frankel, Waterhouse, & Teissedre, 1995; Rodrigo & Bosco, in press; Staško, Polovka, Brezová, Biskupič, & Malí'k, 2006; Zafrilla et al., 2003). Epidemiological studies have repeatedly shown an inverse association between the risk of myocardial infarction and the consumption of wine or the intake level of some particular flavonoids, but no clear associations have found between cancer risk and polyphenols consumption (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005). Phenolic compounds are potent anti-oxidants and exhibit various physiological activities including anti-inflammatory, anti-allergic, anti-carcinogenic, anti-hypertensive, anti-arthritic and anti-microbial activities.

Phenolic compounds may affect growth and metabolism of bacteria. They could have an activating or inhibiting effect on microbial growth according to their constitution and concentration (Alberto, Farías, & Manca de Nadra, 2001, 2002; Alberto, Gómez-Cordovés, & Manca de Nadra, 2004). On pathogenic microorganisms the anti-bacterial

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effect depends of the phenolic compounds and of the strains tested. (Puupponen-Pimiä et al., 2005; Wen, Delaquis, Stanich, & Toivonen, 2003). Rodríguez Vaquero, Alberto, and Manca de Nadra (in press) reported the anti-microbial properties of pure phenolic compounds and polyphenols of different wines against *Proteus mirabilis*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

The food contamination by microorganisms has attracted increased attention because it is a problem that has not yet been brought under adequate control despite the preservation techniques available. *Listeria monocytogenes* has been recognized as an emerging foodborne pathogen and has become a major concern to the food-processing industry and to health authorities over the last decades. It is found in soil, water, dairy products, including soft cheeses, and in raw and undercooked meat, poultry, seafood and related produce.

L. monocytogenes assumed public health significance as a result of its presence in foods linked to several outbreaks of listeriosis. Despite the efforts to eradicate the organism from foods, *L. monocytogenes* contamination continues to occur. It is a common bacterium in environment and animals, and may be transferred to food and human gastrointestinal tract via raw milk and contaminated dairy products. This organism may cause meningitis, sepsis or abortion, but in practice only pregnant women and people with immune defects are in danger of infection (Nester, Roberts, Pearsall, Anderson, & Nester, 1998).

Resistance to anti-microbial agents has become an increasingly important and pressing global problem. So, new classes of anti-microbial drugs are urgently required. In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Rates, 2001). It is generally accepted that phytochemicals are less potent anti-infectives than agents of microbial origin, i.e. antibiotics. However, new classes of anti-microbial drug are urgently required and the flavonoids represent a novel set of leads. Future optimization of these compounds through structural alteration may allow the development of a pharmacologically acceptable anti-microbial agent or group of agents (Cushnie & Lamb, 2005).

The aim of this work was to investigate and compared the anti-microbial properties of pure flavonoid and non-flavonoid phenolic compounds and total polyphenols of three Argentinean wines varieties, Cabernet Sauvignon, Malbec and Merlot against *L. monocytogenes*.

2. Materials and methods

2.1. Microorganism and culture conditions

The bacterium used as test organism, *L. monocytogenes*, was isolated from human infection by public Hospital of Tucumán, Argentina. This bacterium was identified in our laboratory by its biochemical properties. *L. monocytogenes* was grown aerobically at 30 °C in brain heart infusion (BHI) broth and agar (Britania, Argentina) medium, pH 7.0.

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.

2.2. Chemicals

Gallic acid was obtained from Merck (Darmstadt, Germany), catechin was obtained from Sigma (St. Louis, MO), vanillic acid, protocatechuic acid, caffeic acid, quercetin and rutin were purchased from ICN (Ohio, USA). Ciocalteu's phenol reagent and sodium carbonate were from Merck.

2.3. Samples

2.3.1. Pure phenolic compounds

For agar diffusion assays and growth curves, all phenolic compounds were dissolved in ethanol 99.8% and filter-sterilized through a 0.22 μm membrane filter.

2.3.2. Wines

Different Argentinean wines Cabernet Sauvignon, Malbec and Merlot were used. Wine samples were protected against sunlight and stored at 4°. Wines were concentrated in rotary evaporator. Without concentrate, two and four-fold concentrated (1×, 2×and 4×) wines were clarified by the addition of 30, 60 and 120 mg/ml of activated charcoal, respectively, in order to eliminate phenolic compounds. All wine samples were filter-sterilized. Clarified wines were used as controls.

2.4. 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).

2.5. Anti-bacterial activity

2.5.1. Agar diffusion assay

The agar diffusion test was used to investigate anti-bacterial effects of phenolic compounds. Soft agar medium was inoculated with liquid overnight culture to a cell density of 2.0×10^9 cfu/ml, and plates containing 10 ml of agar media were overlaid with 10 ml of this inoculated soft agar. Equidistant holes were made in the agar. Thirty microlitre volumes of each sample were pipetted into the agar wells. Chloramphenicol (1000 mg/l) was used as a positive control and the negative control was ethanol. After 24 h incubation the inhibition zones were measured to an accuracy of 0.5 mm and the effect was calculated as a mean of triplicate tests.

2.5.2. Growth curves in presence of pure phenolic compounds

The liquid growth medium used in this experiment was BHI. The initial pH was adjusted to 7.0. Phenolic

compounds were added to the medium to obtain a final concentration of 25, 50, 100, 200 and 500 mg/l. Ethanol was added to all media to obtain a final concentration of 5% v/v. The media were inoculated 7% with overnight culture. Bacterial growth was followed by incubation for 18 h at 30 °C in a tunable microplate reader (Versamax, Molecular Devices). The plates used were microtitre plate flat form. The cultures were agitated each five minutes. Bacterial growth measurement was determined indirectly by measuring absorbance at 560 nm by the microplate reader and directly by enumerating the number of viable cells by plating serial dilutions in the BHI agar medium.

2.5.3. *L. monocytogenes* growth curves measurement in presence of wine

The fresh growth medium BHI supplemented with 50% Cabernet Sauvignon, Malbec and Merlot wine samples (1×, 2× and 4×) were inoculated with 10% of overnight culture. Bacterial survival was followed by taking samples from the cultures during incubation time. Samples were diluted with physiological solution and the proper dilutions were plated. The plates were incubated as above and the bacterial counts were recorded. The inhibitory effects of different wines on the bacteria were measured by comparing the control growth curves (50% clarified wine and 50% BHI) with those obtained from cultures with wines.

2.6. Statistical analysis

All experiments were carried out at least in triplicate. Statistical analysis was performed using MS-Excel software.

3. Results

3.1. Agar diffusion assays

Table 1 shows the results of the anti-microbial screening of different concentrations of seven pure phenolic compounds: four phenolic acids, gallic, vanillic, protocatechuic and caffeic and three flavonoids, rutin, catechin and quercetin against *L. monocytogenes*. The phenolic compounds were tested at a concentration range from 1 to 1000 mg/l. The microorganism was inhibited by all phenolic compounds and the effect depends on its concentrations. Non-flavonoid compounds presented inhibitory effects from 5 mg/l except for caffeic acid that inhibited from 1 mg/l and for protocatechuic acid that inhibited from 25 mg/l. Caffeic acid at a concentration of 1000 mg/l produced the strongest anti-listerial activity. Between flavonoid compounds, rutin was the only that inhibited the growth of *L. monocytogenes* at all concentrations assayed reaching the strongest effect at only 25 mg/l.

The anti-listerial activities of 1×, 2× and 4× concentrated samples of the three varieties of Argentinean wines, are represented in Fig. 1. Cabernet Sauvignon variety showed an inhibition zone that increased from 4 to 10 mm when the concentration of total phenolic compounds increased from 2300 to 8209 mg/l. Samples of Malbec wine

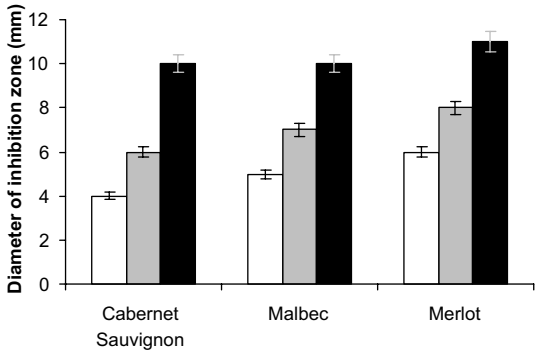

Fig. 1. Anti-microbial activity of total phenolic compounds present in different wines against *L. monocytogenes*. (□) Control wine; (▒) twofold concentrated wine and (■) fourfold concentrated wine. Content in mg/l of polyphenols: Cabernet Sauvignon (□ 2300, ▒ 4594, ■ 8209), Malbec (□ 2522, ▒ 4840, ■ 9393) and Merlot (□ 2704, ▒ 5010, ■ 9883). The values are the average of three determinations.

Table 1
Anti-bacterial activity of phenolic compounds against *L. monocytogenes*

	Phenolic compound concentrations (mg/l)								
	1	5	10	25	50	100	200	500	1000
Non-flavonoids									
Gallic acid	–	W	+	+	+	+	++	++	+++
Vanillic acid	–	W	W	+	+	+	+	++	++
Protocatechuic acid	–	–	–	W	+	++	++	+++	+++
Caffeic acid	+	+	++	++	++	+++	+++	+++	++++
Flavonoids									
Rutin	++	++	+++	++++	++++	++++	++++	++++	++++
Quercetin	–	+	++	++	+++	+++	++++	++++	++++
Catechin	–	+	+	++	++	++	+++	+++	++++

No anti-microbial activity (–), inhibition zone <1 mm. Weakly anti-microbial activity (W), inhibition zone 1 mm. Slight anti-microbial activity (+), inhibition zone 2–3 mm. Moderate anti-microbial activity (++), inhibition zone 4–5 mm. High anti-microbial activity (+++), inhibition zone 6–9 mm. Strong anti-microbial activity (++++), inhibition zone >9 mm.

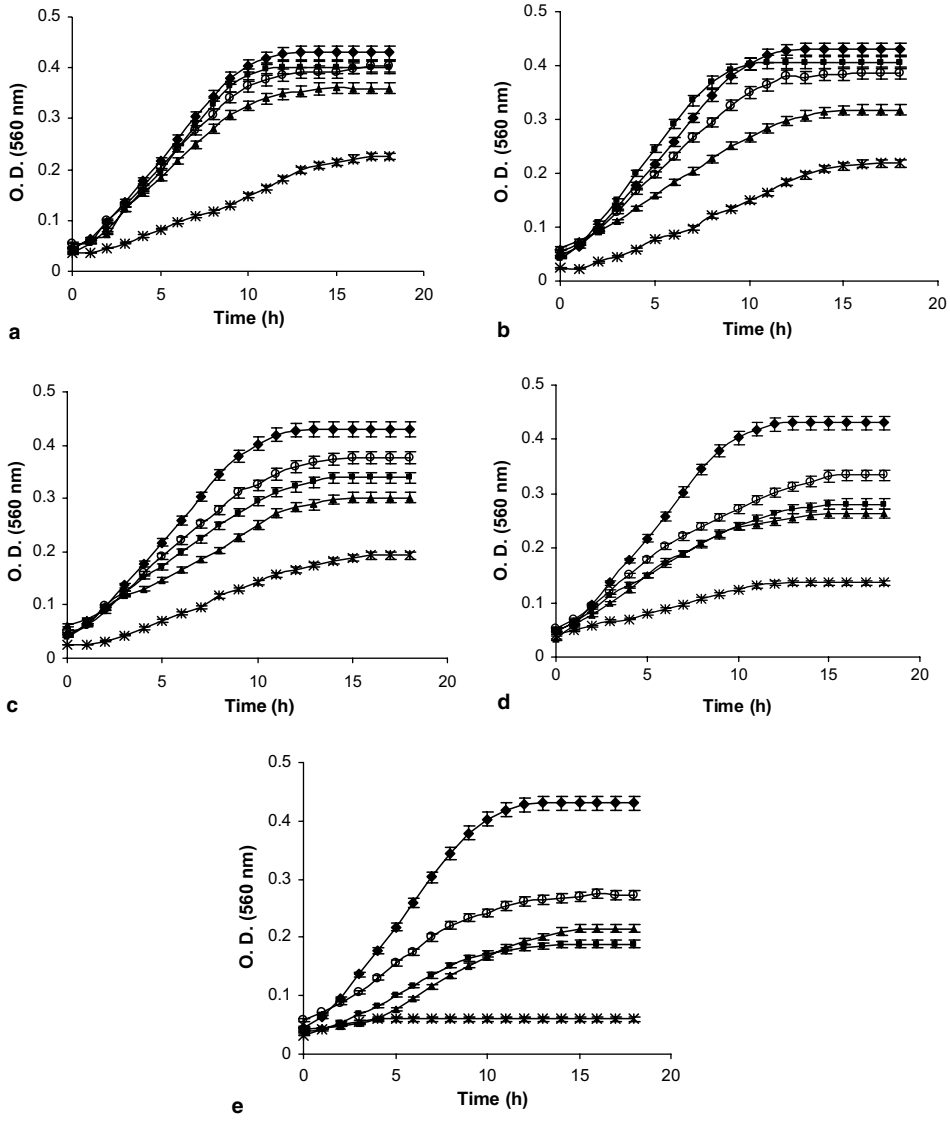

Fig. 2. Growth curves of *L. monocytogenes* in BHI media supplemented with non-flavonoid compounds at: (a) 25 mg/l, (b) 50 mg/l, (c) 100 mg/l, (d) 200 mg/l and (e) 500 mg/l. (◆) Control, (■) gallic acid, (▲) protocatechuic acid, (○) vanillic acid and (✳) caffeic acid. Each point represents the average value of four determinations.

produced an inhibition zone from 5 to 10 mm when the polyphenolic concentration increased from 2522 to 9393 mg/l and Merlot wine samples showed an inhibition zone from 6 to 11 mm when the polyphenolic concentration increased from 2704 to 9883 mg/l. Clarified wines were inactive against *L. monocytogenes*. The results indicate that there is a correlation between the polyphenols concentration and the inhibitory effect.

3.2. Growth curves in presence of pure phenolic compounds

Fig. 2 shows the effect of different concentrations of phenolic acids on *L. monocytogenes* growth. At 25 mg/l (Fig. 2a) gallic and vanillic acids had no effect; protocatechuic acid decreased 16% the final cell density and the maximal growth rate (μ_{max}) and caffeic acid was the most effective compound with a final cell density (end of exponential growth phase) and μ_{max} 31% and 48% lower than the control, respectively.

With 50 mg/l (Fig. 2b), only gallic acid did not modify the growth parameters of the bacterium. An inhibition of 10%, 26% and 33% in the final cell density and 13%, 37% and 49%, in the μ_{max} was observed by the addition of vanillic, protocatechuic and caffeic acids, respectively.

With 100 mg/l (Fig. 2c) the inhibitory effect showed a decrease of 22%, 13%, 30% and 42% in the final cell density and 36%, 21%, 47% and 53% in the μ_{max} in presence of gallic, vanillic, protocatechuic and caffeic acids, respectively. With 200 mg/l (Fig. 2d) an inhibition of 35%, 22%, 39% and 59% in the final cell density and 51%, 24%, 47% and 76%, in the μ_{max} by the addition of gallic, vanillic, protocatechuic and caffeic acids, respectively, was observed. With 500 mg/l

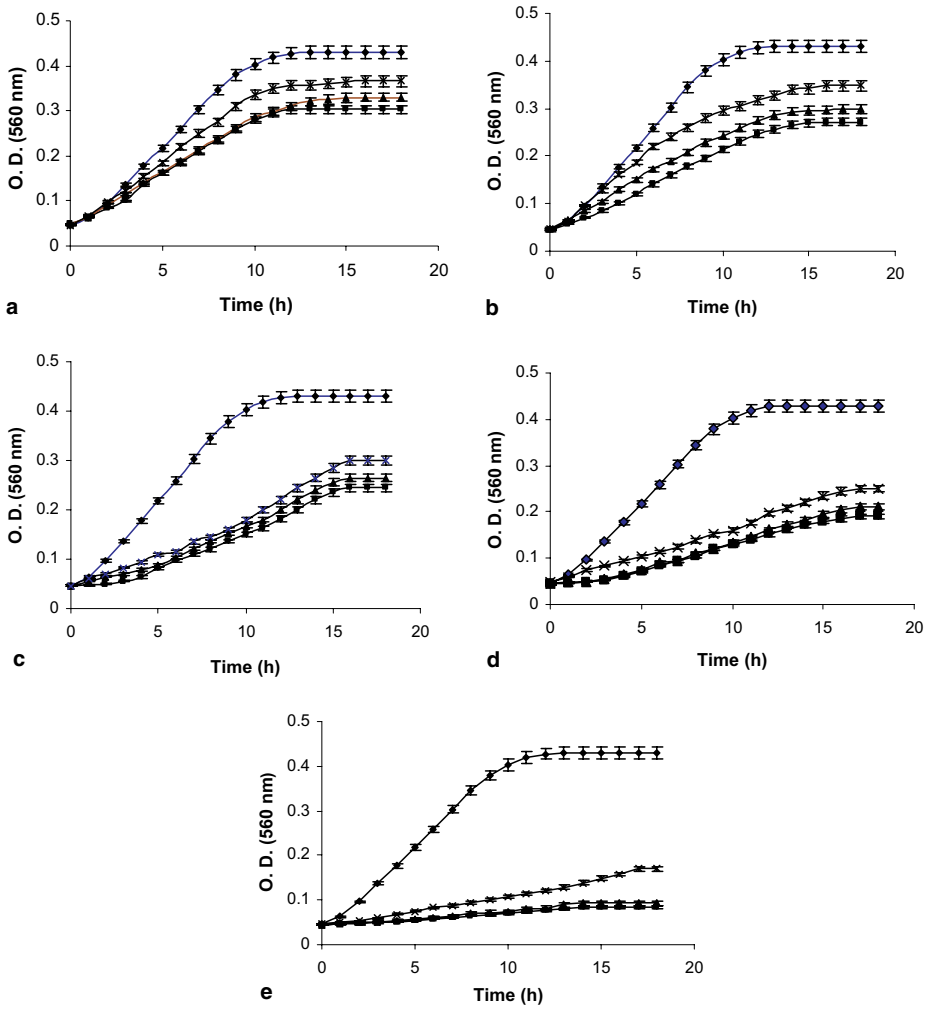

Fig. 3. Growth curves of *L. monocytogenes* in BHI media supplemented with flavonoid compounds at: (a) 25 mg/l, (b) 50 mg/l, (c) 100 mg/l, (d) 200 mg/l and (e) 500 mg/l. (◆) Control, (■) rutin, (▲) quercetin; and (**X**) catechin. The values are the average of four determinations.

(Fig. 2e) a decrease of 56%, 37% and 50% in the final cell density and 56%, 37% and 52% in the μ_{max} in presence of gallic, vanillic and protocatechuic acids, respectively, was observed. The growth of *L. monocytogenes* was totally inhibited only with 500 mg/l of the hydroxycinnamic derivate, caffeic acid.

Fig. 3 shows the growth of *L. monocytogenes* in presence of different concentrations of flavonoid compounds. Rutin, quercetin and catechin at all concentrations decreased the growth parameters of this bacterium. Rutin and quercetin were more effective as anti-listerial compounds than catechin (Fig. 3a–e). In presence of 100 mg/l rutin, quercetin and catechin the inhibition of growth parameters was significantly higher than that observed with the same concentrations of hydroxybenzoic acids (Fig. 3c). The growth of *L. monocytogenes* was totally inhibited with 500 mg/l of rutin and quercetin (Fig. 3e).

With all pure phenolic compounds, the anti-microbial effect was directly related to phenolic compound concentration.

3.3. Viable cell number

Fig. 4 shows that there was no noticeable inhibitory effect on *L. monocytogenes* cell viability in presence of 25 and 50 mg/l gallic and vanillic acids. Protocatechuic and caffeic acids and all flavonoid compounds caused a noticeable decrease of viable cells at all concentrations assayed.

A diminution of two, one and half log cycles was observed in presence of 500 mg/l caffeic acid, rutin and quercetin, respectively. The diminution of cell viability was correlated with phenolic compounds concentration.

3.4. Survey of L. monocytogenes in presence of wine

Fig. 5 shows the viability diminution of *L. monocytogenes* when the BHI medium was added with wine samples.

At 36 h storage, phenolic compounds of Cabernet Sauvignon wine showed a decrease in the number of viable cells of 40%, 54% and 67%, for 1×, 2× and 4× wines,

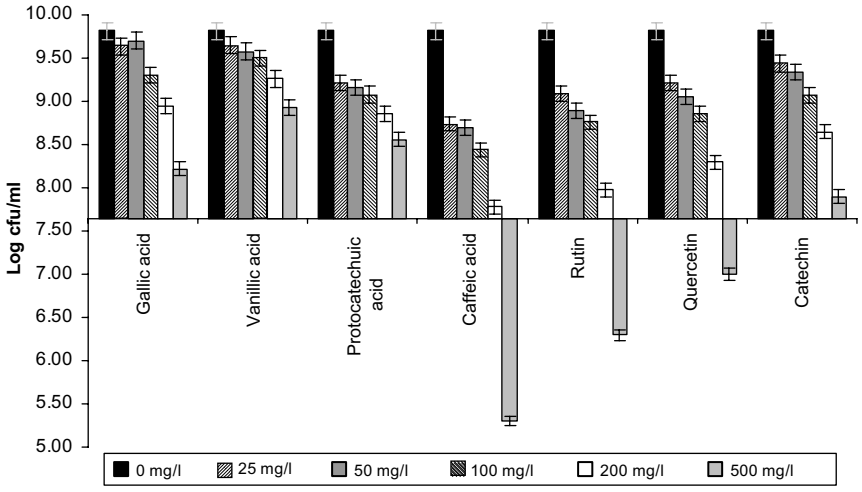

Fig. 4. Number of viable cells of *L. monocytogenes* in BHI media supplemented with different concentration of phenolic compounds. Each point represents the average value of three determinations.

respectively, with respect to the correspondent clarified control wines. The highest inhibition was observed by the addition of Merlot samples to BHI medium. There was a decrease of 50%, 64% and 79% for 1×, 2× and 4× samples of Merlot wines, respectively. In this case the viability was from 15% to 25% and from 11% to 20% lower than Cabernet Sauvignon and Malbec wines, respectively.

The viable cells of *L. monocytogenes* disappeared completely at 60, 72 and 84 h storage in media with wines concentrated 4×, 2× and 1×, respectively. At 84 h in 1× clarified controls the remnant number of viable cells were 1.5×10^3, 2.0×10^3, 3.4×10^3 cfu/ml for Cabernet Sauvignon, Malbec and Merlot wines, respectively.

4. Discussion

Pure phenolic compounds and different concentrations of three wine varieties were evaluated for their effects on the growth of Gram-positive bacterium, *L. monocytogenes*. Between non-flavonoid compounds, the hydroxycinnamic derivative caffeic acid was the more effective to inhibit the growth of *L. monocytogenes*. Hydroxycinnamic acids, due to their propenoic side chain, are much less polar than the corresponding hydroxybenzoic acids and this property might facilitate the transport of these molecules across the cell membrane, which might be related in turn to the stronger inhibitory effect. Campos, Couto, and Hogg (2003) reported that in *Oenococcus oeni* growth the inhibitory effect was stronger with hydroxycinnamic acids than with hydroxybenzoic acids. Puupponen-Pimiä et al. (2001) studying the action of phenolic compounds on Gram-positive and Gram-negative bacteria found that the phenolic cinnamic, coumaric, caffeic and ferulic acids inhibited *Escherichia coli* and *Salmonella enterica* at high concentrations (10 mg/ml).

Wen et al. (2003) found that hydroxycinnamic acids exhibited activity against several strains of *L. monocytogenes*.

Gallic and protocatechuic acids have three and two hydroxyl groups in their structures, respectively, while vanillic acid has one hydroxyl group, and a methoxy group instead of hydroxyl group. As speculation, considering the chemical structure the lower inhibitory effect of vanillic acid with respect to gallic and protocatechuic acid could be related with the presence of the methoxy group instead of hydroxyl group. Rutin, glycoside form of quercetin, was the most effective flavonoid with anti-listerial activity. Rauha et al. (2000) reported that rutin was inactive against *S. aureus*, *Staphylococcus epidermidis*, *Bacillus sudbtilis*, *Micrococcus luteus*, *E. coli* and *P. aeruginosa*.

Several investigations have examined the relationship between flavonoid structure and anti-bacterial activity. Cushnie and Lamb (2005), reported that the anti-bacterial mechanisms of action of various flavonoids could be attributed to inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function or inhibition of energy metabolism.

In this work, all wines samples showed an inhibition zone against the bacterium, which increases when the polyphenolic concentration increased from control wines to fourfold concentrated wines. The controls of clarified wines were inactive against *L. monocytogenes*, indicating that the responsible of the anti-microbial effects were the polyphenolic compounds present in the red wines. Different results about the anti-listerial effect of berries phenolics was reported by Puupponen-Pimiä et al. (2005). The authors reported that *Listeria* strains were not affected by berry compounds.

Our results could be the basis to demonstrate if the wine consumption with a meal may collaborate in the health

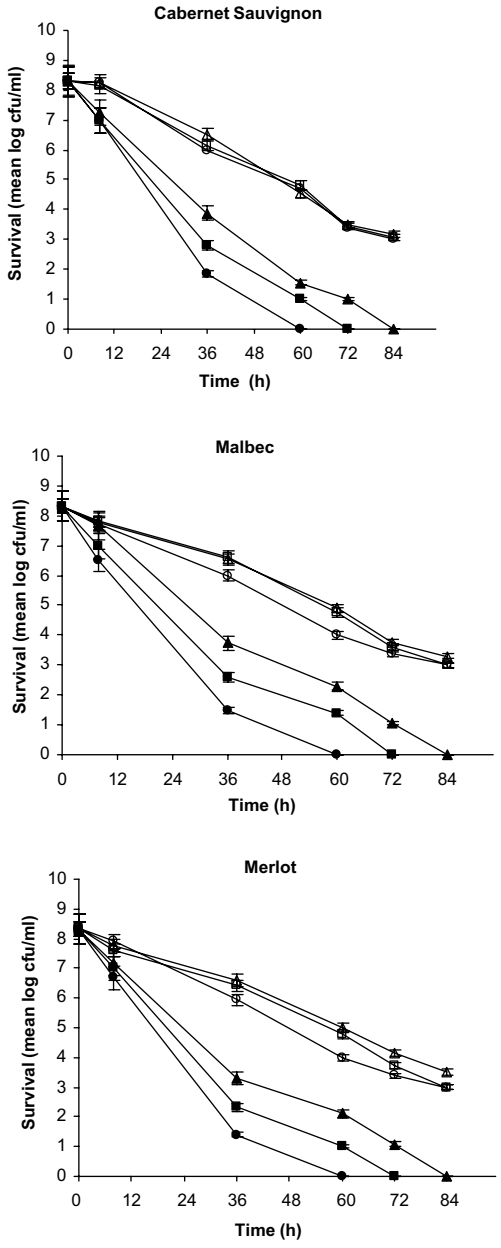

Fig. 5. Survey of *L. monocytogenes* in BHI media supplemented with 50% of wines. Clarified wines: (△) 1× (from 25.1 to 50.3 mg/l GAE), (□) 2× (from 34.9 to 70.4 mg/l GAE) and (○) 4× (from 48.4 to 74.1 mg/l GAE). Wines samples: (▲) 1×; (■) 2× and (●) 4×. Each point represents the average value of three determinations.

protection against some foodborne organisms such as *L. monocytogenes.*

Acknowledgments

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