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Growth Parameter and Viability Modifications of *Escherichia coli* by Phenolic Compounds and Argentine Wine Extracts

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Abstract The anti-bacterial effect of pure non-flavonoids gallic, vanillic, protocatechuic, and caffeic acids and flavonoids quercetin, rutin, and catechin and the effect of total polyphenols of three Argentinean wine varieties, Cabernet Sauvignon, Malbec, and Merlot, against *Escherichia coli*, microorganism frequently detected in fresh and processed foods, was investigated. The hydroxycinnamic derivate caffeic acid and the flavonoid quercetin were the more effective against *E. coli*. The polyphenol effect was ethanol independent. The *E. coli* decimal reduction times were 2.9, 2.1, and 0.65 h for Malbec wine and 2.8, 2.3, and 0.64 h for Merlot wine with respect to 1×, 2×, and 4× concentrated wine samples, respectively. For Cabernet Sauvignon wine, the values were 6.3, 3.7, and 1.28 h for 1×, 2×, and 4× concentrated samples, respectively. With clarified wines, the decimal reduction times were higher with values ranging from 15 to 18.4 h in the wine samples. So the phenolic compounds present in red wines could be considered as an interesting alternative to be used as natural preservative against pathogenic microorganisms.

Keywords Phenolic compounds · Anti-bacterial activity · Wine · Growth inhibition · *Escherichia coli*

Introduction

Vegetarian foods and products have frequently been found to be contaminated with spoilage and pathogenic organisms, notably, *Clostridium sporogenes*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas*, and *Escherichia coli* [1]. The food contamination and spoilage by microorganisms have attracted increased attention because is a problem

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that has not yet been brought under adequate control despite the preservation techniques available.

The exploration of naturally occurring antimicrobials for food preservation receives increasing attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives [2]. So, new classes of antimicrobial drugs are urgently required. [3].

Phenolic compounds are found in fruit, vegetables, nuts, seeds, stems, and flowers as well as tea, wine [4], propolis, and honey [5] and represent a common constituent of the human diet. They have been proposed to have a variety of biological effects on human health, including anti-inflammatory activity, enzyme inhibition, anti-allergic activity, anti-oxidant activity, vascular activity, and cytotoxic anti-tumor activity [6].

Interest in phenolic compounds in wine has increased in recent years because of their potential beneficial effects on human health. Phenolic compounds are responsible for some of the major organoleptic properties of wines, in particular color and astringency.

Rodríguez Vaquero et al. [7] reported that *Proteus mirabilis*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *S. aureus*, and *E. coli* exhibited different sensitivities toward different concentrations of phenolic compounds and wines.

The aim of this work was to investigate the effect of non-flavonoid and flavonoid phenolic compounds and total polyphenols of three Argentinean wine varieties, Cabernet Sauvignon, Malbec, and Merlot on the viability of *E. coli* ATCC 35218, microorganism widely distributed in the environment and frequently detected in fresh and processed foods.

Materials and Methods

Strain Used and Preparation of the Inocula

E. coli ATCC 35218 (ATCC: American Type Culture Collection) was used as test organism. *E. coli* was grown at 37 °C in Nutrient 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.

Preparation of Samples

Pure Phenolic Compounds All phenolic compounds were dissolved in ethanol 99.8% and filter-sterilized through a 0.22 μm membrane filter.

Wines Three varieties of Argentinean wines, Cabernet Sauvignon, Malbec, and Merlot were used. Wines were concentrated in rotary evaporator. Without concentrate, two-, and fourfold 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. Wine samples were protected against sunlight and stored at 4 °C.

Ethanol Determinations

Ethanol concentrations in wine samples were measured enzymatically with alcohol dehydrogenase method (Table 1) [8].

Table 1 Ethanol concentration of each wine sample.

Grape variety	Wine concentration		
	1×	2×	4×
Ethanol concentration (%)			
Cabernet Sauvignon	12	8.3	0.3
Malbec	12	8.9	0.5
Merlot	12	8.5	0.6

Each value represents the average of three determinations.

Chemicals

Catechin was obtained from Sigma (St. Louis, MO, USA), gallic acid was obtained from Merck (Darmstadt, Germany), and vanillic acid, protocatechuic acid, caffeic acid, quercetin, and rutin were purchased from ICN (Ohio, USA). Ciocalteu's phenol reagent and sodium carbonate were obtained from Merck. Alcohol deshydrogenase and nicotinamide adenine dinucleotide were purchased from Sigma.

Colorimetric Determination of Total Phenolic Compounds

Colorimetric determination of total phenolics was based on the procedure of Singleton and Rossi [9]. A standard curve of gallic acid was used. Results are expressed as milligram per liter gallic acid equivalents.

Anti-bacterial Activity

Influence of Pure Phenolic Compounds on the Growth The liquid growth medium used in this experiment was nutrient broth. 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 37 °C in a tunable microplate reader (Versamax, Molecular Devices). The plates used were microtitre plate flat form. The cultures were agitated each 5 min. 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 nutrient agar medium.

The maximum specific growth rate, μ_{max}, was determined by the formula:

μ_{max} (h^{-1})	Ln $[OD_2/OD_1]/t_2-t_1$
OD_2	Absorbance at the end of the exponential phase
OD_1	Absorbance at the beginning of the exponential phase
t_2	Time (h) at the end of the exponential phase
t_1	Time (h) at the beginning of the exponential phase

Influence of Total Polyphenols of Wines on Survival of E. coli The fresh growth medium nutrient broth supplemented with 50% Cabernet Sauvignon, Malbec, and Merlot wine samples (1×, 2×, and 4×) were inoculated with 10% of overnight culture. The initial pH was adjusted to 7.0. 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 wines and 50% nutrient broth) with those obtained from cultures with wines.

Table 2 shows the decimal reduction time (time to reduce the population 90% of its initial value) that were calculated graphically for each wine sample.

Statistical Analysis

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

Results

Influence of Pure Phenolic Compounds on *E. coli* Growth

Figure 1 shows the final cell density and the growth rate of *E. coli* growth after 18 h incubation at 37 °C in presence of different concentrations of phenolic acids. Vanillic and protocatechuic acids (25 mg/l; Fig. 1a) did not modify the growth. Gallic and caffeic acids caused an inhibition of 11% and 36% on the final cell density and 18% and 19% on the maximal growth rate (μ_{max}), respectively. At 50 mg/l (Fig. 1b), only vanillic acid did not modify the growth parameters of the bacterium. An inhibition of 14%, 13%, and 47% in the final cell density was observed by the addition of gallic, protocatechuic, and caffeic acids, respectively. The maximal growth rate decreased 27% with gallic and protocatechuic acids and 31% with caffeic acid.

With the addition of 100 mg/l (Fig. 1c), an inhibition of 25%, 23%, 12%, and 57% in the final cell density and 31%, 23%, 12% and 50% in the μ_{max} in presence of gallic, protocatechuic, vanillic, and caffeic acids was observed, respectively. With 200 mg/l (Fig. 1d), the inhibitory effect increased 36%, 32%, 20%, and 62% in the final cell density and 49%, 41%, 23%, and 66% in the μ_{max} by the addition of gallic, protocatechuic, vanillic, and caffeic acids, respectively. At 500 mg/l (Fig. 1e), gallic, protocatechuic, vanillic, and caffeic acids produced a decrease of 50%, 47%, 30%, and 80% in the final cell density and 65%, 63%, 46%, and 80% in the μ_{max}, respectively. The hydroxycinnamic derivate, caffeic acid, was the strongest to inhibit the growth of *E. coli* at all concentrations.

Table 2 Decimal reduction time calculated graphically for each wine sample.

Grape Variety	Decimal reduction time of *E. coli* (h)		
	Wine concentration		
	1×	2×	4×
Cabernet Sauvignon	6.3	3.7	1.28
Malbec	2.9	2.08	0.65
Merlot	2.81	2.3	0.64
Clarified			
Cabernet Sauvignon	18.4	16.4	15
Malbec	16.9	17.7	15.1
Merlot	17.8	16.3	15.5

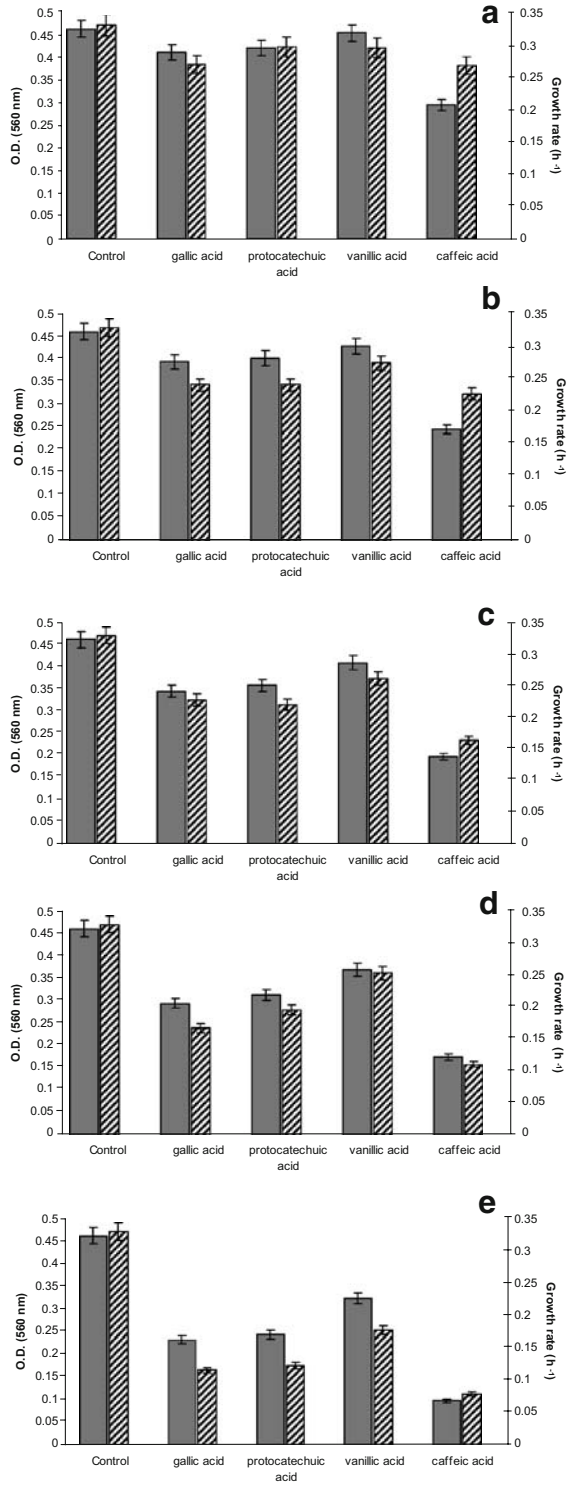

Fig. 1 Final cell density (*shaded bars*) and growth rate (*striped bars*) of *E. coli* ATCC 35218 in nutrient broth media supplemented with non-flavonoid compounds at: **a** 25 mg/l, **b** 50 mg/l, **c** 100 mg/l, **d** 200 mg/l, and **e** 500 mg/l. Each point represented the average value of four determinations

Figure 2 shows the final cell density and the growth rate of *E. coli* in presence of different concentrations of flavonoid compounds. Rutin, quercetin, and catechin at all concentrations decreased the growth parameters. Quercetin and rutin were more effective as antibacterial compounds than catechin (Fig. 2a–e). The growth of *E. coli* was totally inhibited with 500 mg/l of quercetin (Fig. 2e).

In any case, we observed cellular lyses by the phenolic compounds.

Viable Cell Number

Figure 3 shows the number of viable cells at 18 h contact with the different concentrations of phenolic compound.

In control medium, the number of viable cells increased from 2.0×10^7 to 4.5×10^9 cfu/ml. Figure 3 shows that the addition of 25 mg/l of gallic or caffeic acids reduced viable counts by 0.34 and 0.89 log cycle with respect to the control.

Treatment with 50 mg/l of gallic or caffeic acids reduce 0.6 and 1.5 log cycles the number of viable cells compared to the control, respectively. Amounts of 25 and 50 mg/l of vanillic or protocatechuic acids did not decrease significantly the number of viable cells of *E. coli.*

The addition of 100 mg/l of gallic, protocatechuic, vanillic, or caffeic acids decreased the number of viable cells by 0.95, 0.5, 0.27, and 2.15 log cycles, respectively.

From 200 mg/l of caffeic acid, we observed cellular death. Treatment with 200 and 500 mg/l of caffeic acid reduced viable counts by 3.4 and 4.35 log cycles compared with the control.

With respect to flavonoid compounds, a diminution of 1.54, 1.13, and 0.54 log cycles with respect to the control was observed with the addition of 25 mg/l of quercetin, rutin, and catechin, respectively.

Quercetin (200 mg/l) was the only flavonoid compound that produced a diminution of 1 log cycle from the initial cell number inoculated in the media. At 500 mg/l, quercetin or rutin reduced 3 and 1 log cycles of inoculated cells, respectively.

Survey of *E. coli* in Presence of Wine

Figure 4 shows the viability diminution of *E. coli* when the nutrient broth medium was added with wine samples.

At 24 h incubation, Malbec and Merlot control wine samples produced the total inhibition of viable cells of *E. coli* (2.2×10^9 cfu/ml). This effect is achieved with Cabernet Sauvignon control wine sample at 30 h incubation. With the addition of 4× wine samples, the cellular death was produced in 9 h by Malbec and Merlot wines and in 12 h by Cabernet Sauvignon wine. There were no significant differences between the death rates in presence of Malbec and Merlot wines. The total polyphenols of Cabernet Sauvignon wines were less effective to produce the cellular death of *E. coli.*

The ethanol concentrations were 12%, 8%, and <1% for 1×, 2×, and 4× in wines (and clarified samples), respectively. The pH_{wines} values were 3.6±0.05 independently of the concentration and clarification. Both results indicate that the inhibitory effect was independent of the acidity or ethanol concentrations.

From Fig. 4, the decimal reduction time calculated shows that the time to reduce by 90% viable cells of *E. coli* were 2.9, 2.1, and 0.65 h for Malbec wine and 2.8, 2.3, and 0.64 h for Merlot wines with respect to 1×, 2×, and 4× concentrated samples, respectively. For Cabernet Sauvignon wine, the values were 6.3, 3.7, and 1.28 h, for 1×, 2×, and 4×

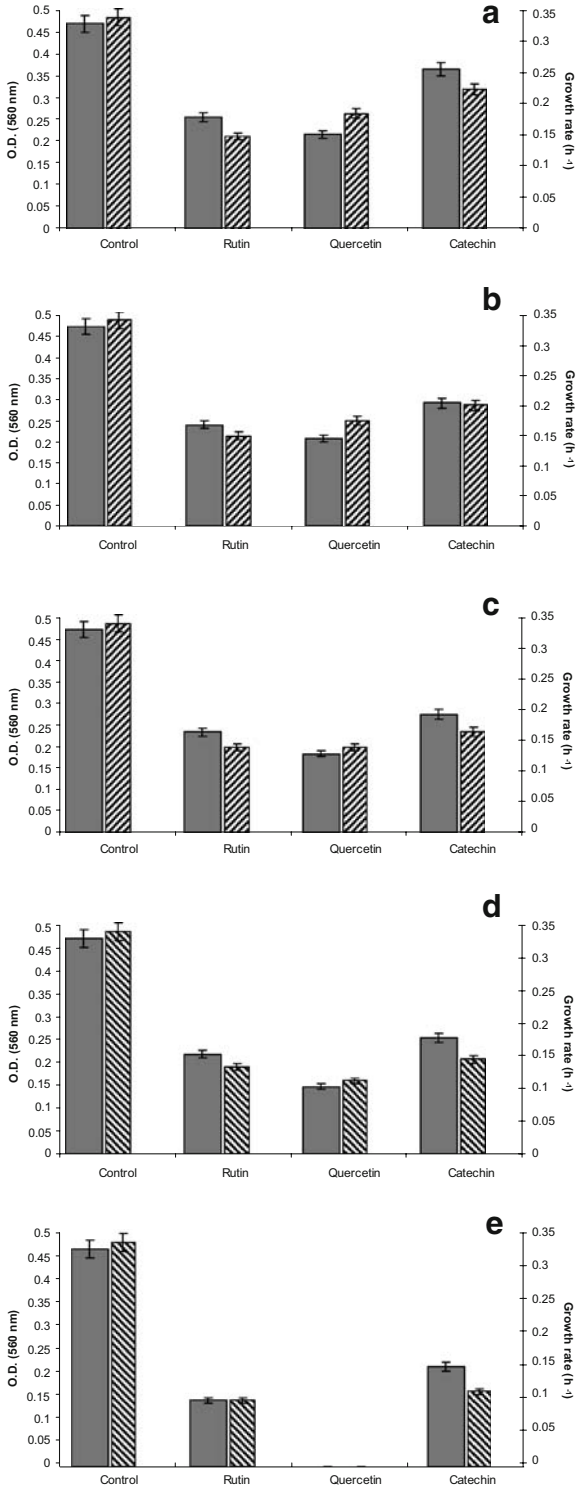

Fig. 2 Final cell density (*shaded bars*) and growth rate (*striped bars*) of *E. coli* ATCC 35218 in nutrient broth media supplemented with flavonoid compounds at: **a** 25 mg/l, **b** 50 mg/l, **c** 100 mg/l, **d** 200 mg/l, and **e** 500 mg/l. The values are the average of four determinations

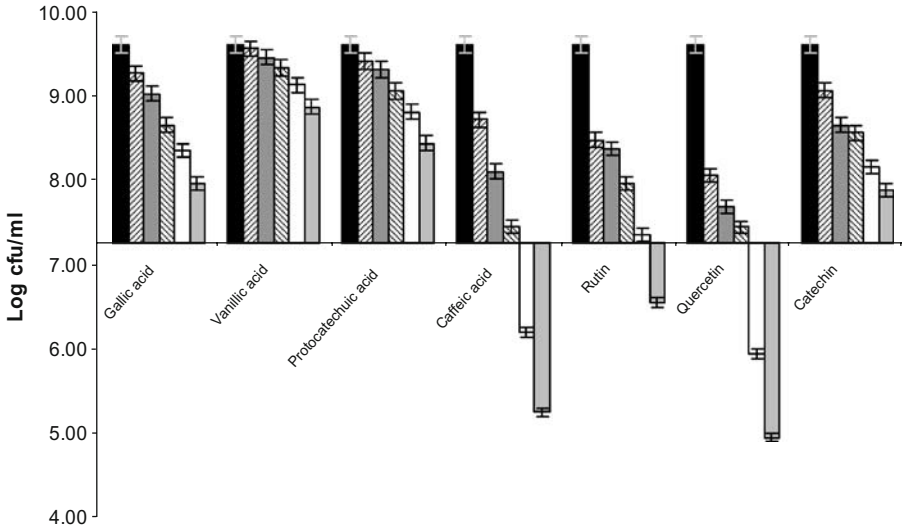

Fig. 3 Log of the number of viable cells (cfu/ml) of *E. coli* ATCC 35218 in nutrient broth media supplemented with different concentration of phenolic compounds: 0 mg/l (*black bars*), 25 mg/l (▨), 50 mg/l (*dark gray bars*), 100 mg/l (▧), 200 mg/l (*white bars*) and 500 mg/l (*light gray bars*). Each point represented the average value of three determinations

concentrated samples, respectively. With clarified wines, the decimal reduction times were higher with values ranging from 15 to 18.4 h in the wine samples.

Discussion

The chemical structure of hydroxybenzoic acids, gallic and protocatechuic acids have three and two hydroxyl groups in their structures, respectively; vanillic acid has only one hydroxyl group and one methoxy group instead of hydroxyl group. As observed in *L. monocytogenes* [10], in this case again, the lower inhibitory effect of vanillic acid with respect to gallic and protocatechuic acids could be related with the presence of hydroxyl groups. The hydroxycinnamic derivative caffeic acid was more effective to inhibit the growth parameters of *E. coli* ATCC 35218 than the hydroxybenzoic acids, as observed for *L. monocytogenes* [10]. However, caffeic acid was more effective to inhibit the growth parameters of the gram-negative *E. coli* ATCC 35218, producing cellular death with 200 mg/l than the inhibitory effect on *L. monocytogenes*, in which cellular death was produced at a caffeic acid concentration of 500 mg/l. Different results were reported by Puupponen-Pïmia et al. [11], demonstrating that the inhibition of *E. coli* by caffeic acid was produced at higher concentrations (50-fold higher). Helander et al. [12] reported that the outer membrane of gram-negative bacteria functions as a preventive barrier against hydrophobic compounds.

All flavonoids were inhibitory for *E. coli.* Quercetin was the most effective. Ohemeng et al. [13] screened 14 flavonoids for inhibitory activity against *E. coli* DNA gyrase and other microorganisms. They found that *E. coli* DNA gyrase was inhibited in different extent by seven of the assayed compounds including quercetin, proposing that the inhibitory activity was due in part to the DNA gyrase inhibition. Mirzoeva et al. [14] demonstrated that

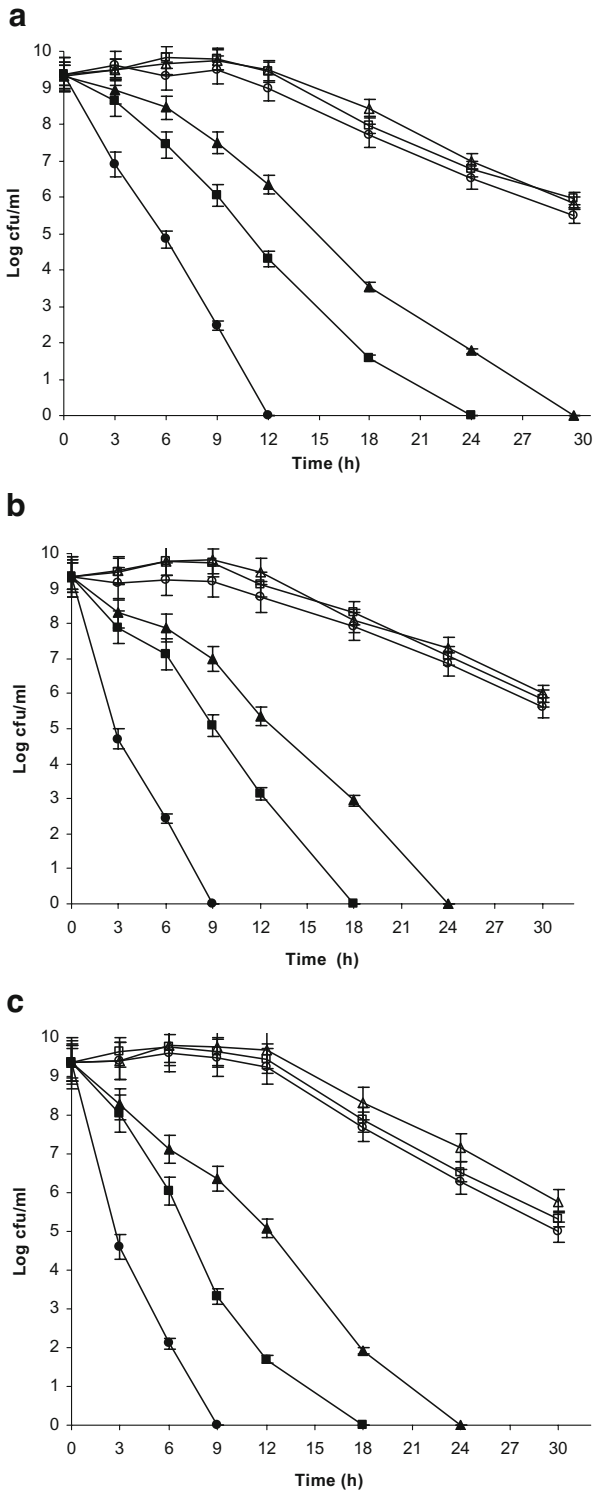

Fig. 4 Survey of *E. coli* in nutrient broth media supplemented with 50% of wines. Clarified wines: 1× (*open triangle*), 2× (*open square*), and 4× (*open circle*). Wine samples: 1× (*closed triangle*), 2× (*closed square*), and 4× (*closed circle*). Content in mg/l of polyphenols in clarified wines: **a** Cabernet Sauvignon (1×, 35.2; 2×, 40.1; 4×, 50.0), **b** Malbec (1×, 25.1; 2×, 34.9; 4×, 48.4), and **c** Merlot (1×, 50.3; 2×, 70.4; 4×, 74.1). Content in mg/l of polyphenols in wine samples: **a** Cabernet Sauvignon (1×, 2,300; 2×, 4,594; 4×, 8,209), **b** Malbec (1×, 2,522; 2×, 4,840; 4×, 9,393), and **c** Merlot (1×, 2,704; 2×, 5,010; 4×, 9,883). Each point represents the average value of three determinations

quercetin caused an increase in permeability of the inner bacterial membrane and a dissipation of membrane potential. This fact disturbed the capacity for ATP synthesis and membrane transport. Bernard et al. [15] found that the glycosylated flavonol rutin inhibited topoisomerase IV-dependent decatenation activity of the *E. coli* strain that is essential for cell survival. Ikigai et al. [16] reported that catechin may perturb the lipid bilayers by directly penetrating them and disrupting the barrier function. Bernard et al. [15] reported that despite amino acid sequence and structural and mechanistic similarities between DNA gyrase and topoisomerase IV, compounds selectively acting as topoisomerase IV poisons and inactive on gyrase do exist.

Rodriguez Vaquero et al. [10] reported the effect of the flavonoid compounds against *L. monocytogenes.* The comparative of the inhibitory effect of quercetin on *L. monocytogenes* and *E. coli* shows that this compound was more effective on *E. coli* producing higher cellular death of the inocula with lower quercetin concentration (200 mg/l). As in both microorganisms, observing the absorbance during the growth, there was no detected cellular lysis by the phenolic compound, it is possible to infer that the DNA gyrase of *E. coli* was more sensitive than that of *L. monocytogenes* to quercetin inhibition. The requirement of a hydroxyl group at the C-3 position, as quercetin, for DNA cleavage activity with DNA gyrase is not necessary for topoisomerase IV. On the other hand, rutin was more effective on *L. monocytogenes* than *E. coli.* Perhaps this fact could be related to the molecular difference with the aglicone quercetin. There is the carbohydrate rutinose instead of the hydroxyl group at the C-3 position.

All wine samples produced bacterial death. The rate of death increased when the polyphenolic concentration increased from control wine to fourfold concentrated wines. The controls of clarified wines were inactive against *E. coli*, indicating that the factors responsible for the anti-microbial effects were the polyphenolic compounds present in the red wine samples. The ethanol concentration in the wine samples and its respective clarified samples were the same, proving that they are not responsible for the antibacterial effect.

Rodriguez Vaquero et al. [10] studied the effect of different polyphenols of the same wine varieties on *L. monocytogenes*, and the values of the decimal reduction time were higher (ranged from 7.6 h to 4.4 h) than those obtained for *E. coli*.

Food contamination is still an enormous public health problem. The actual tendency is the use of natural preservatives. With these investigations, we confirmed that the phenolic compounds present in red wines could be considered as an interesting alternative to be used as natural preservative against pathogenic microorganisms when consumed with a meal.

However, studies in food to confirm the antimicrobial efficiency level of these phenolic compounds are undertaken in our laboratory.

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