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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 of Lactobacilli (Cerela), Chacabuco 145, 4000, Tucumán, Argentina

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Summary

The aims of this work were to investigate the antibacterial effect of phenolic compound combinations and total polyphenols of Argentinean red wines varieties against *Escherichia coli* ATCC 35218 and *Listeria monocytogenes* using commercial fish meat as model food. Rutin-quercetin combination and the three wine varieties produced cellular death of both bacteria on fish meat at 4 ºC. Rutin-quercetin combination was effect even at 20 °C on fish meat. Clarified wines were inactive against both bacteria, indicating that the responsible of observed effect were the polyphenols of wines. 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 during the transport and conservation of fish meat to the fish market, which is an effective antibacterial agent even when there is an Interrupted in the cold chain.

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

[*]Corresponding author; Phone: 54 381 4247 752 ext. 7067; Fax: 54 381 4247 752; E-mail: mariajo@fbqf.unt.edu.ar

Introduction

Food safety is a fundamental concern of 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 a dangerous product and change in the sensory properties, rendering it unsuitable for human consumption.

Listeria monocytogenes is a Gram positive bacterium responsible for the severe food-borne 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 proper at cooking temperature but is capable of growing at refrigeration temperature (*4*).

Escherichia coli is a gram negative bacterium, the primary source of pathogenic bacteria on meat products (*5*), Some strains of *E. coli* can cause diarrhea, urinary tract infections, inflammations and peritonitis in immune-suppressed patients as children and elderly people (*6, 7*). As a consequence, the absence of *E. coli* in foods can be used to asses it the sanitary quality (*8).*

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 safety of some chemical preservatives and negative consumer reaction to preservatives they perceive as chemical and artificial, has prompted an increased interest in more natural alternatives. Hence, there has been recent interest in testing natural products, including plant-derived compounds, for anti-listerial 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, wine (*10*). They have a variety of beneficial effects on human health, including anti-inflammatory activity, anti-allergic activity, anti-oxidant activity and cytotoxic activity (*11*). Phenolic compounds 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, salts; aliphatic and aromatic alcohols, amino acids and phenolic compounds are present at much lower concentrations. The phenolic composition of wine is determined initially 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 posses antibacterial activity (*14, 15, 16, 17*), but the exact mechanisms responsible for the antimicrobial activity of wine 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 accentuating the role of non-phenolic constituents of wine, such us organic acids, ethanol, etc. (*19*).

We have previously found that *Proteus mirabilis, Serratia marcescens, Klebsiella pneumoniae, Pseudomonas aeruginosa, S. aureus, E. coli* and *L. monocytogenes* exhibited different sensitivities towards different concentrations of phenolic compounds and wines in standard laboratory media (*14, 15, 16*). Later we demonstrate that the use of wine phenolic compounds as natural biopreservatives for bovine meat was effective to reduce the viability of *E. coli* and *L. monocytogenes* in a food system model (*20*).

At the present, no reports regards to antibacterial effect of phenolic compounds on fish meat model are present in the scientific literature, the majority information has been conducted in laboratory media, and consequently little is understood about their effectiveness when applied to fish meat.

The aim of this work was investigated the antibacterial efficiency of three phenolic compounds combinations and total phenolic compounds of three Argentinean red wines varieties on *E. coli and L. monocytogenes* viability in a fish-meat model system, at 4 °C and 20 °C.

Materials and Methods

Strain used and preparation of the inocula

The bacteria used as test organism were *Listeria monocytogenes,* isolated from human infection by public Hospital of Tucumán, Argentina and *Escherichia coli ATCC 35218* (American Type culture collection). *L. monocytogenes* was grown aerobically at 30 ºC in brain heart infusion (BHI) broth (Britania, Argentina) medium, pH 7.0. *E. coli* was grown at 37 ºC 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 *Listeria 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, 50000 UI, acriflavine HCL 0.0025 UI, ceftazidime, 0.01UI. The medium used for enumeration of *E. coli* in meat was Mc Conkey 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.

Samples

Pure phenolic compounds

Gallic acid was obtained from Merck (Darmstadt, Germany), protocatechuic acid, caffeic acid, quercetin and rutin were purchased from ICN (Ohio, USA). The purity level of all phenolic compounds was > 98 %. All phenolic compounds were dissolved in ethanol 99.8 % (Merck, Darmstadt, Germany) and filter-sterilized through a 0.22 µm membrane filter (Durapore, EM PVDF.Millipore). The selected phenolic compounds combinations used were: gallic-protocatechuic acids, gallic-caffeic acids and quercetin-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. 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. Clarified wines were used as controls, without phenolic compounds. Wine samples were protected against sunlight and stored at 4 ºC. The total phenolic compounds, phenolic acids, flavonoids and flavonols concentrations of the three wines used, were determined in a previous work (*14*, *20*).

Antibacterial activity on fish meat model system

Effect of pure phenolic compounds combinations

Lean meat, obtained from a commercially local, was stored at -20 °C. Ten gram of meat was aseptically placed in stomacher bags and ten milliliters of isotonic solution with phenolic compounds combinations were added to the food to obtain a final concentration of 100 or 200 mg/L in a ratio of (1:1). The selected combinations for this experiment were: gallic-protocatechuic acids (G-P), gallic-caffeic (G-C) acids and quercetin-rutin (Q-R). The stomacher bags were inoculated with 10^9 CFU/mL of *E. coli* and were homogenized for 3 min. Stomacher bags were stored at 4 °C or 20 °C for 21 d. The control was the inoculated meat in the stomacher bag added with ten milliliters of isotonic solution with ethanol 5 %.

Effect of polyphenols of wines

Ten gram sample of lean 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 total 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 inoculums. Stomacher bags were stored at 4 °C or 20 °C for 21 d. The survivors of *L. monocytogenes* or *E. coli* were enumerated at different time intervals 0, 4, 7, 14 and 21 d. The samples were serially diluted with isotonic solution and spread on Palcam agar or Mc Conkey 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 wine. 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

The time to reduce by 90 % the viable cells of *L. monocytogenes* or *E. coli* 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. Growth experimental data means were compared using Student's *t*-test.

Results

Survey of E. coli and L. monocytogenes in fish meat added with phenolic compounds combinations

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

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

At 4 °C, in control fish meat *E. coli* growth 0.08 logaritmic cycles at 21 d of incubation. With 100 mg/L, all phenolic compounds combinations produce the death of the bacterium, being G-C and Q-R combinations the most effectives and the lowest *D* value (1.9 d) was found with R-Q combination. At 21 d, 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 d, with the lowest *D* value.

In a control fish meat model, without phenolic compounds added, *L. monocytogenes* increased 3.87 logarithmic cycles the number of cells, at 21 d incubation at 20 °C. At 4 °C *L. monocytogenes* increased 0.60 log cycles the number of viable cells at the end incubation. Table 2 shows the reduction in the number of viable cells of *L. monocytogenes* in fish meat added with phenolic compounds combinations, at 21 days of storage at 20 °C and 4 °C. At 20 °C, 100 mg/L of G-P, G-C and Q-R produce growth inhibition (35.9 %, 75.5 % and 90.7 %, respectively), without cellular death. With 200 mg/L all combinations produce cellular death; Q-R combination was the most effective, with a *D* value 3 and 1.2 fold lower than *D* values found with G-P y G-C,

respectively. At 4 °C, with 100 mg/L all combinations produce cellular death, G-C and Q-R showed the lowest *D* values. The addition of 200 mg/L increased the cellular death, at 14 d no bacteria were detected in fish meat added with G-C and Q-R combinations.

Survey of E. coli and L. monocytogenes in fish meat added with three wines varieties

Figure 1 shows the growth of *E. coli* in fish meat supplemented individually with the three wine varieties, at 20 °C. In control meat (without wine samples addition) *E. coli* increase in 3.98 logaritmic cycles the cell population at 21 d. 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 37.8 %, 75.9 % and 52.03 %, respectively the *E. coli* growth with respect to control meat at 21 d. A diminished of 46.4 %, on the growth of the bacteria was observed with the addition of 200 mg/L of total polyphenols from Cabernet Sauvignon wine as regard to control meat (Fig. 1b). Malbec and Merlot wines produce the death of the inoculated cell, being Malbec wine the most effective, with a lowest *D* value (table 3).

At 4 °C, (Fig. 2), in control meat, *E. coli* growth 0.07 log cycles at 21 d and was similarly to the growth observed with the individual addition of clarified wines. Treatment with 100 mg/L or 200 mg/L of polyphenols of the three varieties produces the cellular death, Malbec wine was the most effective with the lowest *D* values (table 3).

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

At 4 °C (Fig. 4), *L. monocytogenes* growth 0.60 log cycles at 21 d of incubation. Treatment with 100 or 200 mg/L of Cabernet Sauvignon, Malbec and Merlot wines produced cellular death. With 200 mg/L of Merlot or Malbec wines no viable cells were detected at 14 and 21 d, respectively. The lowest *D* value (1.3 d) was observed with polyphenols of Merlot wines (table 4).

Discussion

In this study we investigated the antibacterial activity of phenolic compounds combination and total polyphenols of three red wines varieties on a fish meat model system, against *E. coli and L. monocytogenes*, bacteria frequently detected in meat, with economic impact in the food industry. The wines used in this investigation were the traditionally produced in Argentine and consumed widely around the world. Also, the influence of temperature on the antibacterial activity was investigated and the relation between the phenolic compound content in each wine variety and the antibacterial activity was studied.

As expected, even when at 20 ºC there was an important inhibitory effect, the combinations of phenolic compounds were more effectives at 4 ºC, producing cellular death at the two concentrations assayed, as evidenced by the values of decimal reduction times, corresponding the lowest to rutin-quercetin combination. This combination is also effective at 20 °C, with 200 mg/L produce cellular death of both bacteria; this fact is a great discovery since it could prevent contamination of the fish meat against these pathogenic bacteria, without the need to use cold. This is a big finding for the transport of fish meat, during which can cut the cold chain at various times and for the conservation during the storage of this meat.

With respect the antibacterial effect of polyphenols of wines, the best inhibitory effect of wine phenolic compounds against *L. monocytogenes* and *E. coli* viability on fish meat was observed with Merlot and Malbec wine varieties, at 4 °C. The differences observed in the antibacterial effect could be related to the differences in phenolic compounds concentration and composition between the wine varietals studied. In previous work, Rodríguez Vaquero et al. (*14, 20*), reported that total phenolic compound, flavonoid and flavanol compounds concentration were greater in Merlot and Malbec wines compared with Cabernet Sauvignon variety. Besides, Rodríguez Vaquero et al. (*15, 16*), reported that flavonol compounds, such as rutin and quercetin, were the compounds with best antibacterial activities in a culture medium. Merlot and Malbec wines content higher concentration of flavonol compounds than Cabernet Sauvignon wines, this could be related with the major antibacterial activity observed with these wines.

The clarification was effective to remove phenolic compounds of the three wines, there were no significantly differences in ethanol concentration or pH between wines and clarified wines; so control meats were added with clarified wines and they were inactive against both bacteria,

indicating that the responsible of the antibacterial effects were the phenolic compounds present in wines.

Papadopoulou et al. (*23*) indicates that some phenolic acids of wines are probably the most active components in inhibiting the growth of Gram-positive and Gram-negative bacteria and yeasts. Boba et al. (*18*) observed that the antibacterial activity of wines could not be related to their total phenolics and resveratrol content, antioxidant capacity, ethanol content, or pH. They indicate that 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 ability 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 °C than at 20 °C, and its mode of action depends on migration into bacterial membranes (*26*), which are less fluid at chill temperatures.

A group of ten colleagues determine that not significantly sensorial changes in fish meat were evidenced at the phenolic compound concentrations used in this study. To corroborate these results, studies of sensorial evaluation are carried out by professional and qualified panelists.

Conclusion

The use of wine phenolic compounds as antibacterial agents with refrigerated temperature could be a good combination to prevent fish meat contamination and extend the shelf life of the product. Besides, would provide additional benefits inherent to their natural biological properties and health benefits. Futhermore, the used of rutin – quercetin combination as preservative compound is a big finding for the transport and conservation of fish meat to the fish market to obtain a safe product.

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Table 1

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

Phenolic compounds Combinations	Log cycles reduction of *E. coli*											
	20 °C						4 °C					
	100 mg/L			200 mg/L			100 mg/L			200 mg/L		
	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

C: Log cycles reduction with respect to control. **I:** Log cycle reduction with respect to inoculums. ***D***: Decimal reduction time (days). **(-):** No Inhibition observed. *****At 14 d no viable cells are detected.

Table 2

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

Phenolic compounds Combinations	Log cycles reduction of *L. monoytogenes*											
	20 °C						4 °C					
	100 mg/L			200 mg/L			100 mg/L			200 mg/L		
	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

C: Log cycles reduction with respect to control. **I:** Log cycle reduction with respect to inoculums. ***D***: Decimal reduction time (days).**(-):** No Inhibition observed. *****At 14 d no viable cells are detected.

Table 3

Decimal reduction times of *E. coli* calculated graphically for each wine sample at 4 °C.

Wine samples	**Decimal reduction time of *E. coli* (days)**	
	100 mg/L	200 mg/L
Cabernet Sauvignon	7.20	3.10
Malbec	2.60	1.90
Merlot	3.80	2.40

Table 4

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

Wine samples	Decimal reduction time of *L. monocytogenes* (days)	
	100 mg/L	200 mg/L
Cabernet Sauvignon	13.40	6.00
Malbec	6.00	2.00
Merlot	3.70	1.00

Figure legends

Figure 1: Survey of *E. coli* in fish meat supplemented with wines storage at 20 °C: (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.

Figure 2: Survey of *E. coli* in fish meat supplemented with wines at 4 °C: (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.

Figure 3: Survey of *L. monocytogenes* in fish meat supplemented with wines storage at 20 °C: (a) 100 mg/L and (b) 200 mg/L. (♦) Control, Wines: (▲) Merlot (■) Malbec and (x) Cabernet Sauvignon. Clarified wines: (♦) Merlot, (ж) Malbec and (+) Cabernet Sa uvignon. Each point represents the average value of three determinations.

Figure 4: Survey of *L. monocytogenes* in fish meat supplemented with wines at 4 °C: (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.

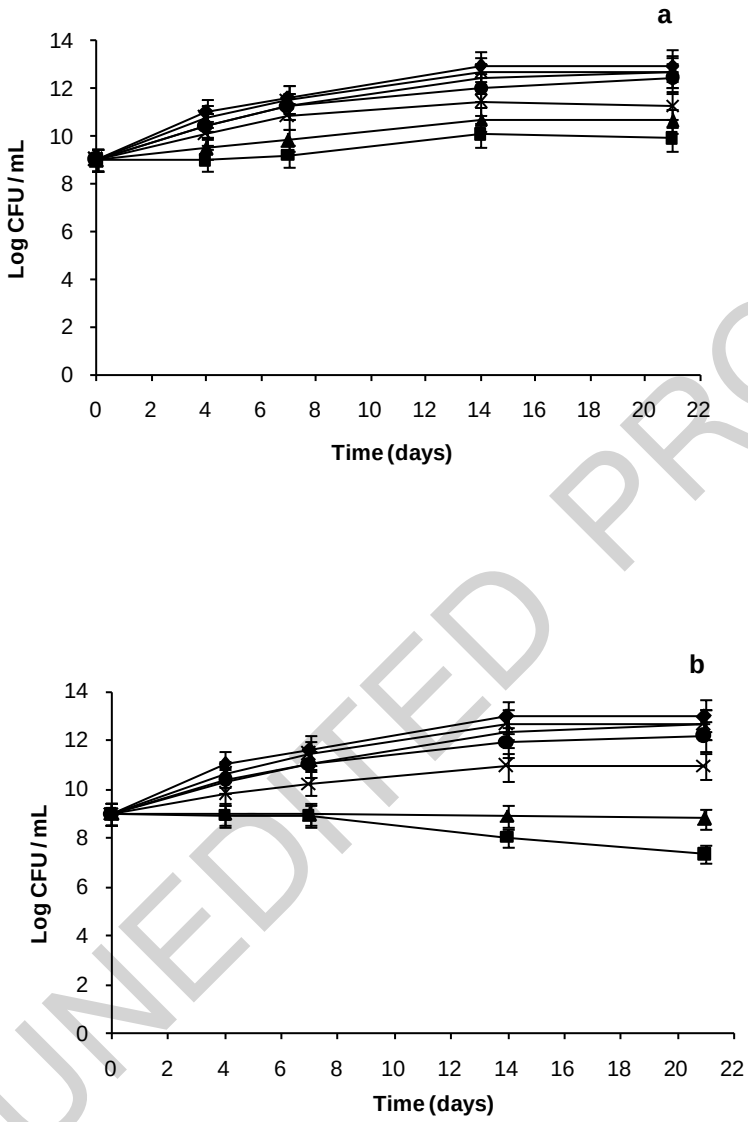

Figure 1

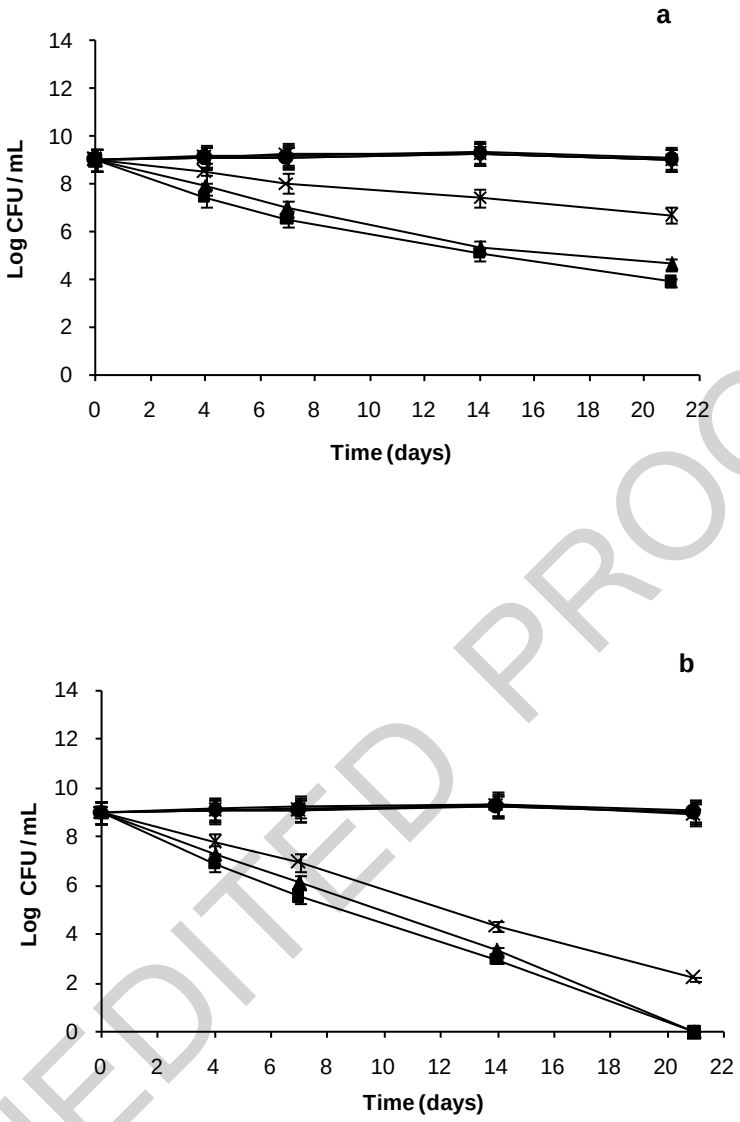

Figure 2

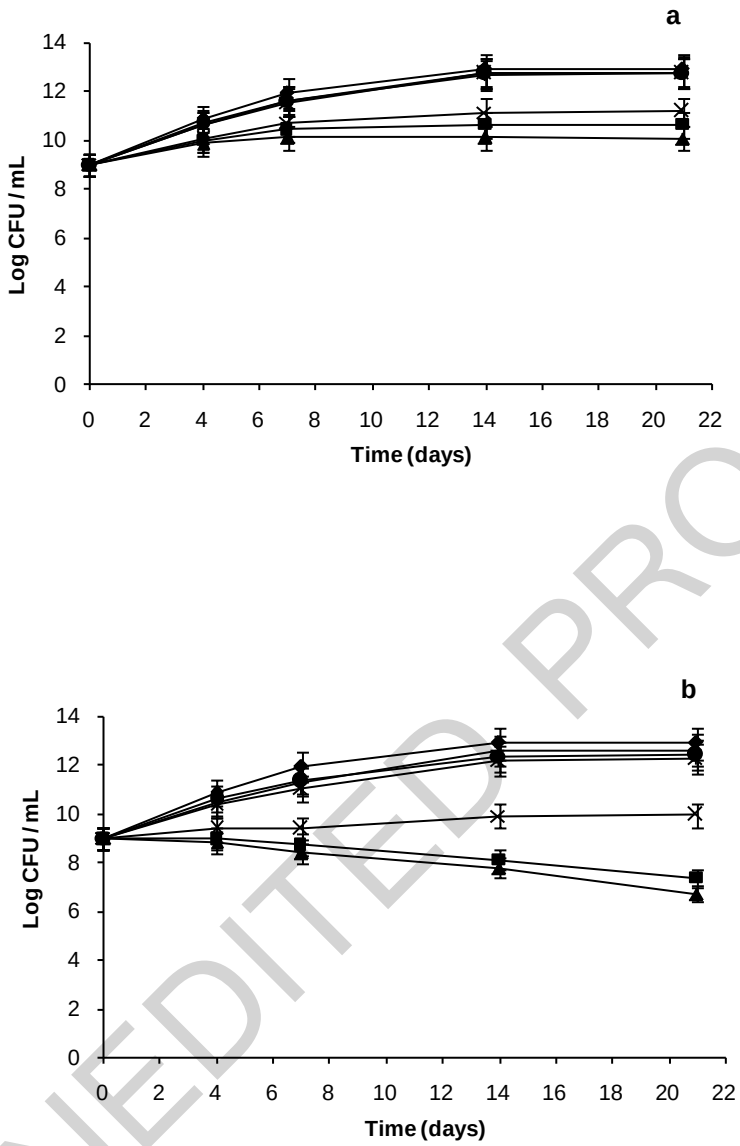

Figure 3

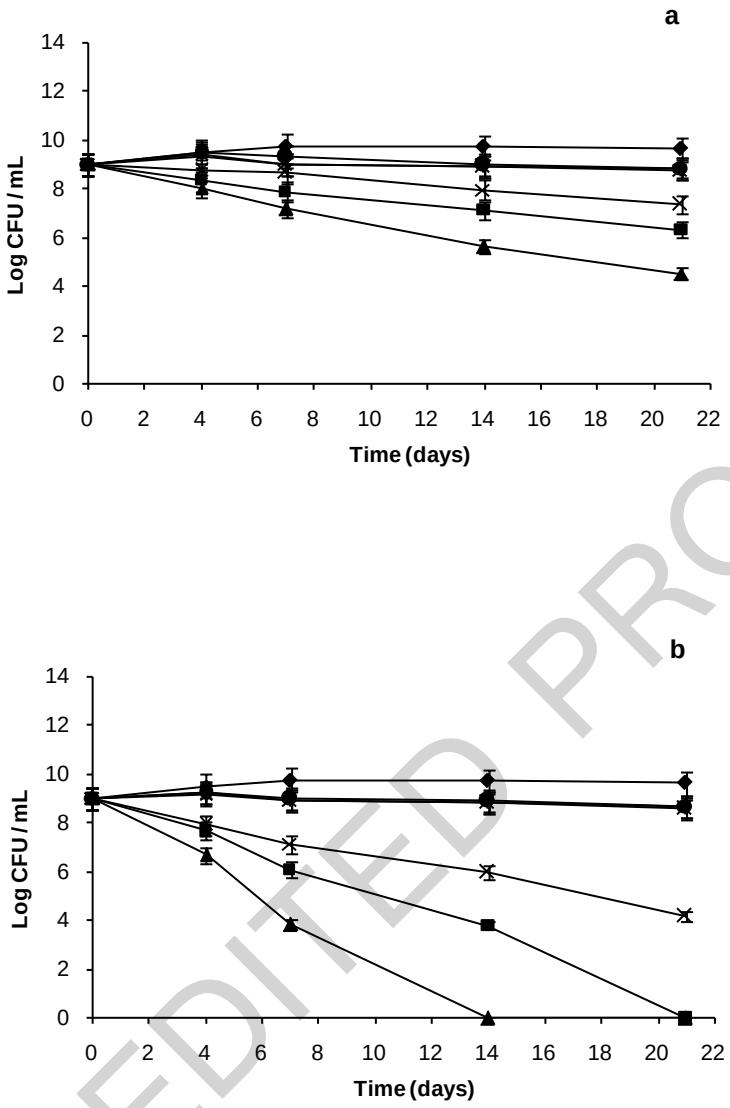

Figure 4