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Effect of Phenolic Compound Mixtures on the Viability of *Listeria monocytogenes* in Meat Model

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Summary

The aim of this work is to investigate the synergistic antibacterial effect of phenolic compound mixtures against *Listeria monocytogenes* in brain heart infusion (BHI) medium, and to select the best mixture for testing their antibacterial activity in a meat model system. In BHI medium, the most effective mixtures were those of gallic and caffeic acids, gallic and protocatechuic acids, and rutin and quercetin. At the concentration of 200 mg/L, the mixtures of gallic and protocatechuic, then gallic and caffeic acids, and quercetin and rutin reduced the number of inoculated cells. At the concentration of 100 mg/L, only the quercetin and rutin mixture produced the same synergistic effect. These combinations were selected for testing in meat. At 20 °C, 100 mg/L of gallic and protocatechuic, then gallic and caffeic acid, and rutin and quercetin mixtures decreased the growth of *L. monocytogenes*, as compared to the control. The inhibitory effect of gallic and protocatechuic acid mixtures increased at the concentration of 200 mg/L. The death of inoculated cells was observed in the treatment with 100 mg/L of all combinations at 4 °C. With the addition of 200 mg/L of these combinations, the lethal effect increased. Gallic and caffeic acid, and rutin and quercetin were the most effective mixtures since after 14 days of incubation no viable cells of *Listeria monocytogenes* were detected. The lowest decimal reduction times of 1.0 and 0.95 day were found for gallic and caffeic acid, and rutin and quercetin mixtures, respectively. These results demonstrate that phenolic compound mixtures have synergistic antilisterial effect with an important bacterial reduction in meat. Therefore, it is possible to search for strategies to combine the synergistic antimicrobial effects of phenolic compounds with their natural biological properties.

Key words: phenolic compounds, *L. monocytogenes*, meat, synergistic effect

Introduction

Food-borne illnesses resulting from the consumption of food contaminated with pathogenic bacteria have been of vital concern for public health. Unfortunately, there is a dramatic increase throughout the world in the number of reported cases of food-borne illnesses (*1*). Fresh meat and derivative products can be easily contaminated with microorganisms and, if not properly handled and preserved, it supports the growth of spoilage and pathogen bacteria, leading to the loss of quality and potential public health problems. Refrigeration storage is usually the most common preservative method for fresh meat and meat products. In order to extend refrigerated storage time, antimicrobial and antioxidant additives, especially of synthetic origin, are added to muscle foods (*2*). The use of chemical additives is perceived by consumers as a health risk. Thus, the exploration of natural

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antimicrobials for food preservation receives increased attention due to the consumers' awareness of natural food products and a growing concern about the microbial resistance to conventional preservatives (*1,3*). As a response to these conflicting demands, current trends in the food industry include the investigation of alternative inhibitors to ensure food safety. 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 a variety of beneficial effects on human health, including anti-inflammatory activity, enzyme inhibition, antiallergic activity, antioxidant activity, vascular activity and cytotoxic antitumour activity (*6*). Phenolic compounds are subdivided into three groups: non-flavonoids, flavonoids and tannins. The non-flavonoids are derivatives of hydroxycinnamic acid (*e.g.* caffeic acid) and of hydroxybenzoic acid (*e.g.* gallic, protocatechuic and vanillic acids). Flavonoids have the flavane nucleus, consisting of two benzene rings (A and B) linked by an oxygen-containing pyrene ring. Based on the saturation and oxidation of heterocyclic ring, flavonoids are subdivided into flavan, flavanone, flavone, flavonol, dihydroflavonol, flavan-3-ol, flavan-4-ol, and flavan-3,4-diol. Tannins can be divided into hydrolysable and condensed tannins.

The antibacterial effects of phenolic compounds on pathogenic microorganisms in food were previously reported by several authors (*7,8*). Rodríguez Vaquero *et al.* (*9*) established that different concentrations of wine phenolic compounds exhibited different antibacterial activity against several bacterial pathogens. Rodríguez Vaquero *et al.* (*10*) and Rodríguez Vaquero and Manca de Nadra (*11*) demonstrated that among the non-flavonoids, the hydroxycinnamic acid derivative, *i.e.* caffeic acid, was more effective as an antibacterial agent against *Listeria monocytogenes* and *Escherichia coli* than hydroxybenzoic acid derivatives, whereas rutin and quercetin were the best antibacterial flavonoids. *L. monocytogenes* can be found in a wide variety of raw and processed foods and in a wide range of environmental conditions (*12*). Meat and meat products have all been associated with *Listeria* contamination; the elderly, pregnant, newborn and immunocompromised populations are more susceptible to listeriosis (*13*). This microorganism cannot survive proper cooking temperature but is capable of growing at refrigeration temperature (*14*). The economic impact due to massive product recalls, severity of the disease, hospitalization and treatment costs has drawn the attention of researchers towards the development of preventive measures to control the spread of *L. monocytogenes* (*15*).

The aim of this work is to investigate the synergistic antibacterial effects of flavonoid and non-flavonoid compound mixtures against *L. monocytogenes* growth in a complex medium in order to select the most effective combination, and to investigate the antilisterial activities of the most effective mixtures of phenolic compounds using meat from the supermarket as model food.

Materials and Methods

Strain used and preparation of inocula

The bacterium used as test organism, *Listeria monocytogenes*, was isolated from human infection at the public hospital of Tucumán, Argentina. This bacterium was identified in our laboratory based on its biochemical properties. *L. monocytogenes* was grown aerobically at 30 °C in brain heart infusion (BHI; Britania Laboratories, Buenos Aires, Argentina) medium, pH=7.0. 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.

Selective medium

The selective medium used for enumeration of *Listeria monocytogenes* in meat was Palcam medium (Britania Laboratories) containing (in 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 and lithium chloride 15.0. The medium was supplemented with (in IU/g): Polymyxin B 50 000, Acriflavine HCL 0.0025, Ceftazidime 0.01 (Britania Laboratories).

Phenolic compounds

Catechin was obtained from Sigma (St. Louis, MO, USA), gallic acid was obtained from Merck (Darmstadt, Germany), vanillic, protocatechuic and caffeic acids, and quercetin and rutin were purchased from I.C.N. Pharmaceuticals (Bryan, OH, USA). All phenolic compounds were dissolved in 99.8 % ethanol and filter sterilized through a 0.22-μm membrane filter.

Inhibitory effect of phenolic compound mixtures in BHI medium

Phenolic compounds for the different combinations were selected considering the antibacterial effect previously determined (Table 1) when assayed as individual compounds in BHI medium (*10*). Phenolic compounds were added to the medium to obtain a final concentration of 100 mg/L (50 mg/L of each one) or 200 mg/L (100 mg/L of each one) of the mixtures. These concentrations were selected because they are naturally found in fruits and wines. The following mixtures of non-flavonoids were utilized: gallic and protocatechuic, gallic and vanillic, gallic and caffeic, protocatechuic and vanillic, and protocatechuic and caffeic acids. The mixtures of flavonoids utilized were: quercetin and rutin, rutin and catechin, and quercetin and catechin. Ethanol was added to all media to obtain a final concentration of 5 %. The media were inoculated with an overnight bacterial culture to obtain $4.37 \cdot 10^7$ CFU/mL. The control was carried out with 5 % ethanol added to the medium, without phenolic compounds and inoculated with an overnight culture. *L. monocytogenes* grown in BHI medium with different phenolic compound mixtures was incubated for 18 h at 30 °C in a tunable microplate reader (VersaMax, Molecular Devices Inc., Sunnyvale, CA, USA) on the flat microtitre plates. The cultures were shaken every 5 min. The samples were serially diluted with isotonic solution, then 0.1 mL of each dilution was spread on nutrient or BHI agar. Plates were incubated for 24 h before enumeration. Each experiment was repeated at least three times.

Table 1. Individual antibacterial effect of phenolic compounds in BHI medium

	γ(phenolic compounds)/(mg/L)			
	50		100	
	Total growth log CFU/mL	Growth reduction log CFU/mL	Total growth log CFU/mL	Growth reduction log CFU/mL
control	2.18	–		
gallic acid	2.06	0.12	1.66	0.52
protocatechuic acid	1.52	0.66	1.44	0.74
vanillic acid	1.94	0.24	1.86	0.32
caffeic acid	1.06	1.12	0.80	1.38
rutin	1.26	0.92	1.12	1.06
quercetin	1.42	0.76	1.22	0.96
catechin	1.70	0.48	1.43	0.75

Theoretical and experimental inhibitory effect of phenolic compound mixtures

Experimental values represent the viable cells (log CFU/mL) obtained after 18-hour incubation in the presence of phenolic compound mixtures. Theoretical values represent the expected values considering the sum of these individual values for each combination (Table 1) and expressed as viable cells (log CFU/mL):

$$\text{Theoretical value} = \text{final growth in control medium/(log CFU/mL)} - \Sigma(\text{growth reduction of individual compounds/(log CFU/mL)}) \quad /1/$$

Inhibitory effect of phenolic compound mixtures can be additive, synergistic or antagonistic. In the first, there is no difference between theoretical and experimental values. In synergism, the experimental value is higher than the theoretical value, while the antagonistic effect is observed when the theoretical value is higher than the experimental value.

Antilisterial effect of the selected phenolic compound mixtures in meat

Lean meat, obtained from a local market, was stored at –20 °C. A mass of 10 g of meat was placed aseptically in a stomacher sterile bag and 10 mL of isotonic salt solution with phenolic compound mixtures were added to the meat in a ratio of 1:1 to obtain a final concentration of 100 or 200 mg/L. The selected mixtures for this experiment are: gallic and protocatechuic acids, gallic and caffeic acids, and quercetin and rutin. The stomacher bags were inoculated with 10^9 CFU/mL of overnight culture and were stomached for 3 min. Stomacher bags were stored at 4 or 20 °C for 21 days. The control was the stomacher bag with the inoculated meat and 10 mL of isotonic salt solution with 5 % ethanol.

The time to reduce the viable cells of *L. monocytogenes* (decimal reduction time) by 90 % was calculated from the slope (Fig. 1) 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 experimental growth data were compared using Student's *t*-test.

Results

Effect of phenolic compound mixtures in BHI medium

In control medium, the number of viable cells increased from $4.37 \cdot 10^7$ to $6.6 \cdot 10^9$ CFU/mL at the end of incubation time (18 h). Fig. 2 shows the number of viable cells of *L. monocytogenes* after 18 h of incubation in BHI medium suplemented with 100 or 200 mg/L of non-fla-

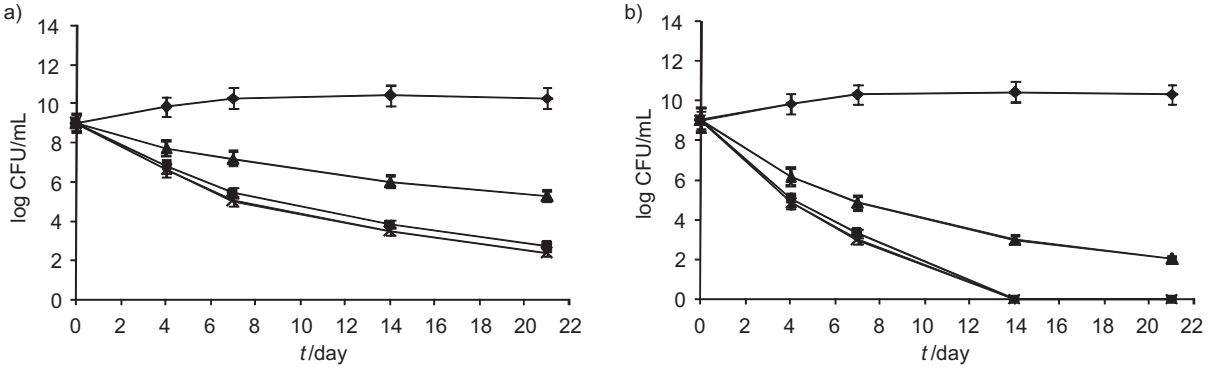

Fig. 1. Survey of *L. monocytogenes* in meat supplemented with phenolic compound mixtures at 4 °C: a) 100 and b) 200 mg/L. (◆) Control, (▲) gallic and protocatechuic acids, (■) gallic and caffeic acids and (x) rutin and quercetin. Each point represents the average value of three determinations

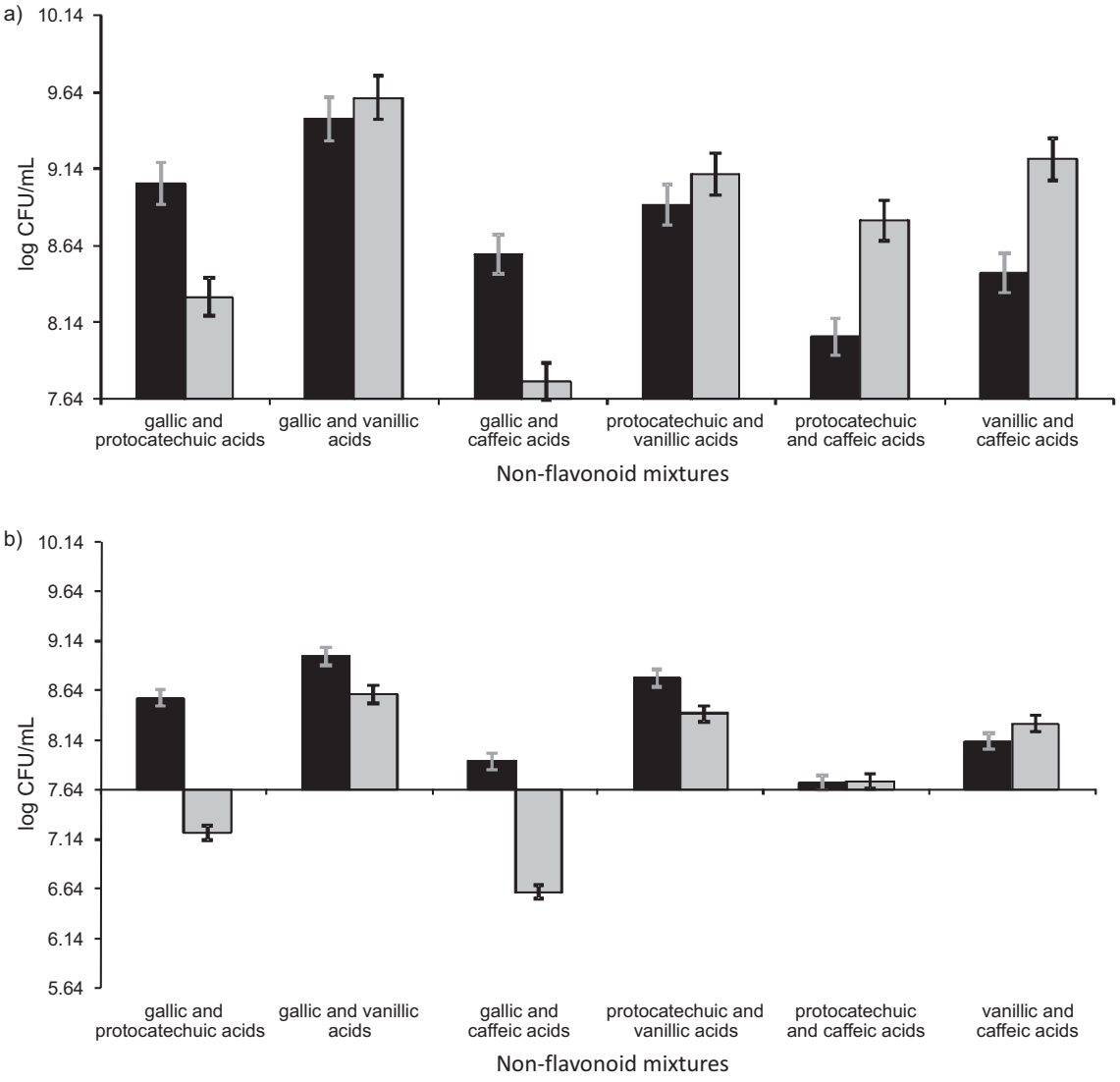

Fig. 2. Number of viable cells of *Listeria monocytogenes* in BHI medium supplemented with: a) 100 or b) 200 mg/L of different non-flavonoid compound mixtures. (■) Theoretical values, (■) experimental values. Each point represents the average value of three determinations

vonoid mixtures. At the concentration of 100 mg/L of non-flavonoid mixtures (Fig. 2a), gallic and protocatechuic, and gallic and caffeic acids were the most effective, demonstrating a synergistic effect. In these cases the viability of microorganism diminished by 0.75 and 0.83 log cycles respectively, with respect to the corresponding theoretical value. Mixtures of gallic and vanillic acids, and protocatechuic and vanillic acids showed an additive effect. At the concentration of 200 mg/L (Fig. 2b), the mixtures of gallic and protocatechuic, and gallic and caffeic acids reduced the number of inoculated cells by 0.44 and 1.04 log cycles, respectively. At this concentration, the inhibitory effect of the mixtures of gallic and vanillic acids, and protocatechuic and vanillic acids shifted to synergy. The most effective non-flavonoid compound mixture was that of gallic and caffeic acids, which significantly ($p<0.01$) reduced *L. monocytogenes* viability at both concentrations.

Fig. 3 shows the inhibition of *L. monocytogenes* viable cells after 18 h of incubation in BHI medium with 100 or 200 mg/L of flavonoid mixtures. At a concentration of 100 mg/L of flavonoid mixtures (Fig. 3a), only the quercetin and rutin mixture had a synergistic effect, reducing the number of inoculated cells by 0.14 log cycles ($p<0.01$). Rutin and catechin, and quercetin and catechin mixtures produced an antagonistic inhibitory effect. With the addition of 200 mg/L of the quercetin and rutin mixture (Fig. 3b), the synergistic inhibitory effect increased, decreasing the number of inoculated cells by 1.54 log cycles. At this concentration, rutin and catechin, and quercetin and catechin mixtures had additive effect, without the reduction of the inoculated cells.

Survival of L. monocytogenes in meat

Fig. 4 shows the growth response of *L. monocytogenes* in meat supplemented with the selected phenolic compound mixtures during storage at 20 °C. In control meat, without phenolic compounds, the CFU of *L. monocytogenes* increased by 3.26 log cycles after 21 days of incubation. At the concentration of 100 mg/L (Fig. 4a),

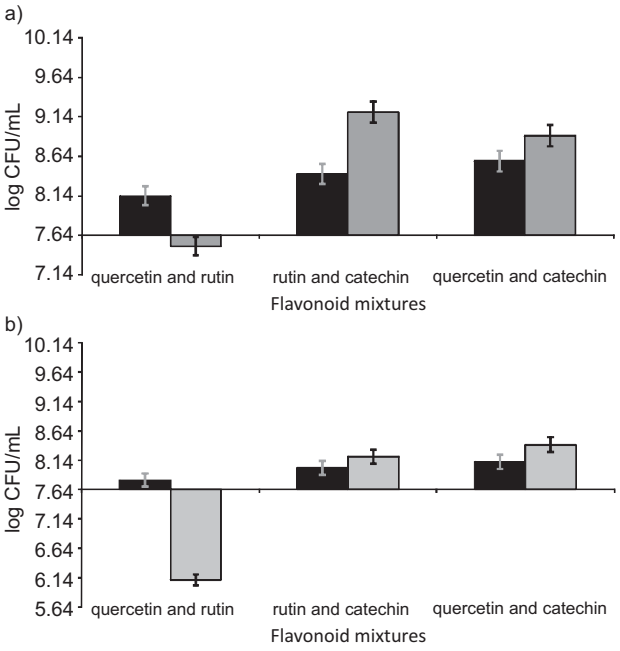

Fig. 3. Number of viable cells of *Listeria monocytogenes* in BHI medium supplemented with: a) 100 or b) 200 mg/L of different flavonoid compound mixtures. (■) Theoretical values, (■) experimental values. Each point represents the average value of three determinations

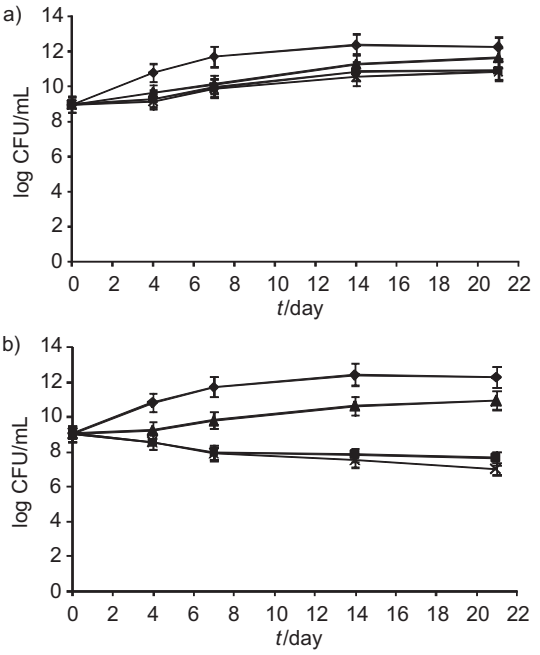

Fig. 4. Survey of *L. monocytogenes* in meat supplemented with phenolic compound mixtures at 20 °C: a) 100 and b) 200 mg/L. (◆) Control, (▲) gallic and protocatechuic acids, (■) gallic and caffeic acids and (x) rutin and quercetin. Each point represents the average value of three determinations

gallic and protocatechuic acids, gallic and caffeic acids, and rutin and quercetin mixtures decreased the growth of *L. monocytogenes* by 18.4, 40 and 42 %, respectively. The addition of 200 mg/L (Fig. 4b) of gallic and protocatechuic acids increased the inhibitory effect by 41.4 % at the end of incubation. Under these conditions, gallic and caffeic acids, and rutin and quercetin mixtures reduced the number of inoculated cells by 1 and 2 log cycles, respectively ($p<0.01$).

The growth response of *L. monocytogenes* in meat supplemented with phenolic compound mixtures at 4 °C is shown in Fig. 1. In control meat, the CFU of *L. monocytogenes* increased by 1.3 logs after 21 days of incubation. At this temperature, all chemical mixtures at the concentration of 100 mg/L (Fig. 1a) reduced the inoculated cells. A reduction of 3.7, 6.3 and 6.7 log cycles was found with gallic and protocatechuic acids, gallic and caffeic acids, and rutin and quercetin mixtures, respectively, after 21 days of incubation ($p<0.01$). The addition of 200 mg/L (Fig. 1b) of these mixtures increased the inhibitory effect. The combination of gallic and protocatechuic acids reduced the inoculated cells by 7 log cycles after 21 days. Gallic and caffeic acids, and rutin and quercetin were the most effective mixtures, since after 14 days of incubation no viable cells of *L. monocytogenes* were detected.

Table 2 shows the decimal reduction times (times to reduce by 90 % inoculated viable cells of *L. monocytogenes*) calculated graphically from Fig. 1 for each phenolic compound mixture at 4 °C. The lowest decimal reduction times corresponding to 1.0 and 0.95 days were found for gallic and caffeic acids, and rutin and quercetin mixtures, respectively.

Table 2. Decimal reduction times of *Listeria monocytogenes*

Mixture of phenolic compounds	Decimal reduction time*/day	
	γ(mixture)/(mg/L)	
	100	200
gallic and protocatechuic acids	3.10	1.40
gallic and caffeic acids	1.80	1.02
rutin and quercetin	1.60	0.95

*calculated graphically for each phenolic compound mixture at 4 °C

Discussion

Among several non-flavonoid mixtures assayed, gallic acid combined with protocatechuic or caffeic acids acted in synergy in the inhibition of *L. monocytogenes* at two assay concentrations (100 and 200 mg/L) at 30 °C. Although the reduction of inoculated cells was observed only in the presence of 200 mg/L of either combination, the gallic and caffeic acid mixture showed greater lethal effect on *L. monocytogenes* than the gallic and protocatechuic acid mixture. Antagonistic effect was obtained with the mixtures of protocatechuic and caffeic acids, and vanillic and caffeic acids. Our results suggest that the observed effect was due mainly to protocatechuic or vanillic acid effect, blocking the action of caffeic acid. All these compounds have similar polarity, and the same capacity to cross the cell membrane. However, protocatechuic and vanillic acids are smaller than caffeic acid, so they can pass through the membrane quickly and probably join the same receptors as caffeic acid. Additive effect was

observed with protocatechuic and vanillic acid mixture, suggesting that the presence of one of these acids does not modify the effect of the other, but they each act independently, probably due to their actions on different receptors. Further studies need to be done to clarify the mechanisms involved in these interactions that lead to synergistic effects.

The most effective antilisterial mixture of flavonoids was quercetin and rutin. This mixture had a synergistic inhibitory effect and reduced the number of inoculated cells of *L. monocytogenes* at two assayed concentrations (100 and 200 mg/L) at 30 °C in culture medium. The three phenolic compound mixtures that were the most effective against *L. monocytogenes* in BHI medium were tested in a meat food system. At the two assayed concentrations at 4 °C, the most effective mixtures were quercetin and rutin, and gallic and caffeic acids, as evidenced by the values of decimal reduction times. The same mixtures were also the most effective under the adverse storage conditions at 20 °C. At present, little information is available about the synergy of phenolic compounds in relation to antibacterial activity. Our results are in agreement with those reported by Arima *et al.* (*16*). They demonstrated the synergy between flavonoids with antibacterial activity and showed that the combinations of quercetin and quercitrin, quercetin and morin, and quercetin and rutin were much more active than either flavonoid alone. Flavonoids have been reported to enhance the anticancer (*17*) or antiviral activity of drugs (*18*).

In the present study, the antimicrobial effects of phenolic compound mixtures in meat were more effective when incubated at 4 than at 20 °C, reducing the viability of *L. monocytogenes* at two concentrations (100 and 200 mg/L) with all mixtures.

Conclusion

The use of phenolic compounds as antimicrobial agents can provide additional benefits. Having in mind the antimicrobial effect of phenolic compounds, it is possible to search for strategies to combine the synergistic antimicrobial effects of phenolic compounds with their natural biological properties. These results may allow the formulation of new antimicrobial products for potential use as food preservatives.

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