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Effects of Natural Phenolic Compounds on the Antioxidant Activity of Lactoferrin in Liposomes and Oil-in-Water Emulsions

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The effect of natural phenolic compounds on the antioxidant and prooxidant activity of lactoferrin was studied in liposomes and oil-in-water emulsions containing iron. The antioxidants tested with lactoferrin were α-tocopherol, ferulic acid, coumaric acid, tyrosol, and natural phenolic extracts obtained from three different extra-virgin olive oils and olive mill wastewater. The natural extracts of olive oils and mill wastewaters were composed mainly of polyphenols and simple phenolics, respectively. Lipid oxidation at 30 °C was determined by the formation of hydroperoxides and fluorescent compounds resulting from oxidized lipid interactions. All phenolic compounds showed synergistic properties in reinforcing the antioxidant activity of lactoferrin in lipid systems containing iron. The highest synergistic effects were observed for the phenolic extracts rich in polyphenols of extra-virgin olive oils and lactoferrin. This synergistic effect was higher in liposomes than in emulsions.

KEYWORDS: Lactoferrin; natural antioxidants; extra-virgin olive oil; olive mill wastewater; synergism; lipid oxidation; emulsions; liposomes; hydroperoxides; fluorescent compounds

INTRODUCTION

Lactoferrin is an iron-binding glycoprotein that belongs to the transferrin family and is present in most exocrine secretions of mammals, including milk. Lactoferrin has been ascribed many biological properties, including regulation of iron absorption (*1*), regulation of the immune system (*2*), growth promotion of lymphocytes (*3*), inhibition of pathogenic bacteria (*4*), and an important role in the iron absorption for breast-fed infants (*5*).

The antioxidant properties of lactoferrin have been demonstrated in various biological and chemical environments (*6*−*8*). This antioxidant activity was shown previously to depend on the lipid system, the concentration, buffer, and the presence of metal ions and oxidation time (*9*). In systems containing high metal concentrations or when the concentration of iron exceeded the chelating ability, the lactoferrin protein showed either low antioxidant efficiency or prooxidant activity (*10*, *11*).

The need of supplementing some foodstuffs with iron motivated our studies of the synergistic effects between lactoferrin and other natural antioxidants. In multicomponent systems, antioxidants may reinforce each other by cooperative effects resulting in an increase of antioxidant activities (*12*). Significant synergism has been observed between preventive antioxidants and chain-breaking antioxidants because they can reduce both the initiation and propagation reactions of lipid oxidation. Lactoferrin can act as a metal chelating agent because of its ability to bind two atoms of iron, and phenolic compounds such as tocopherols or phenolic acids may reinforce this activity. Extra-virgin olive oil phenolic and polyphenolic compounds have demonstrated strong antioxidant activities (*13*−*15*) which have been correlated to oil stability (*16*, *17*). They have recently attracted strong interest for their use as natural additives in foods (*18*).

Lactoferrin also shows bacteriostatic and bactericidal activities related to the iron deprivation of bacteria and the interaction of its N-terminal basic peptide with bacterial phospolipid membranes, respectively (*19*, *20*). The combined antioxidant and antimicrobial properties of lactoferrin have attracted strong interest, and it was demonstrated to be a useful supplement for some foods enriched in iron such as infant formula (*21*). This work was aimed at determining whether antioxidants such as α-tocopherol and other natural phenolic compounds may act synergistically in reinforcing the antioxidant activity of lactoferrin. The potential synergistic or additive effects were determined in liposomes and oil-in-water emulsions containing iron oxidized at 30 °C. The effects of combined antioxidants were evaluated by determining the formation of hydroperoxides based on conjugated dienes and fluorescent compounds resulting from oxidized lipid interactions.

MATERIALS AND METHODS

Materials. Bovine lactoferrin, 90% purity and 20% iron saturation, was obtained from Sigma (St. Louis, MO). Soybean lecithin (40% L-α-phosphatidylcholine), potassium phosphate monobasic, potassium phosphate dibasic monohydrate, the Folin−Ciocalteu reagent, α-toco-

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pherol (95% purity), *o*-coumaric acid, *p*-coumaric acid, ferulic acid, syringic acid, *trans*-cinnamic acid, caffeic acid, *p*-hydroxyphenyl acetic acid, vanillic acid, and protocatechuic acid were also obtained from Sigma. Tyrosol was obtained from Aldrich (Madrid, Spain). Ferrous sulfate heptahydrate and trifluoroacetic acid were obtained from Fluka (New-Ulm, Swizerland). All chemicals and solvents used were either analytical or HPLC grade (Ridel-Haën, Seelze, Germany). Commercially available corn oil was treated to remove natural tocopherols (*22*).

Three extra-virgin olive oils (EVO1, EVO2, and EVO3) were obtained commercially from different geographical regions in Spain and were used for the extraction of phenolic compounds. The Instituto de la Grasa (CSIC, Sevilla) kindly provided olive mill wastewater prepared by a three-phase centrifugation process.

Preparation of Phenolic Extracts from Oils and Mill Wastewater. Phenolic compounds were extracted from oils by using methanol and water (*23*). The phenolic compounds from mill wastewater were extracted using a previous acidification (pH 2), a washing step with hexane, and an extraction in ethyl acetate according to Brenes et al. (*24*). Total phenol content was determined by the Folin−Ciocalteu method (*25*) and expressed as gallic acid equivalents (GAE).

Phenol Composition by High-Performance Liquid Chromatography (HPLC). Total phenolic extracts were analyzed by reversed-phase HPLC (*26*). Peaks were identified by comparing their relative retention times with those of standards. Major peaks corresponding to complex phenols (hydrolyzable phenols) were assigned to the structures previously identified (*27*−*29*) on the basis of their relative elution and ^{1}H NMR spectroscopy (*26*). Results were expressed as gallic acid using an external calibration and given as percentage of total phenolics.

Tocopherols. Tocopherols were analyzed in extra-virgin olive oils by reversed-phase HPLC (*30*).

Preparation of Liposome Samples. Liposomes containing 1% lecithin were prepared in 25 mM phosphate buffers at pH 6.6 as previously described (*9*). Liposome samples were introduced into 50-mL Erlenmeyer flasks (total volume 10 mL), and bovine lactoferrin, ferrous sulfate, and the different phenolic antioxidants were added.

Preparation of Emulsions. Oil-in-water emulsions containing 1% lecithin and 10% corn oil stripped of tocopherols were prepared in 25 mM phosphate buffers at pH 6.6 as previously described (*9*). Emulsions were introduced into 50-mL Erlenmeyer flasks (total volume 10 mL), and bovine lactoferrin, ferrous sulfate, and the different phenolic antioxidants were added.

Oxidation. Triplicate samples of liposomes and emulsions were oxidized with shaking at 30 °C. Oxidative stability was evaluated by measuring conjugated diene hydroperoxides and fluorescence compounds. Inhibition of oxidation was calculated during the propagation period of controls. The induction period (IP) was calculated as the time (in days) required for a sudden change in the rate of oxidation by the method of tangents to the two parts of the kinetic curve (*12*, *31*). Synergism was calculated by comparing the induction periods of formation of fluorescent compounds according to Frankel (*12*).

$$\%\ \text{synergism} = \frac{100 \times [\text{IP (antiox}_1 + \text{antiox}_2) - (\text{IP antiox}_1 + \text{IP antiox}_2)\ ]}{(\text{IP antiox}_1 + \text{IP antiox}_2)}$$

Measurement of Conjugated Diene Hydroperoxides. Weighed liposomes or emulsion samples (100 mg) were dissolved in methanol and ethanol, respectively, and absorbance was measured at 234 nm (Perkin-Elmer UV−Vis spectrophotometer) and calculated as mmol hydroperoxides/kg of oil as described previously (*32*). Duplicate analyses were performed for each sample.

Measurement of Fluorescence Compounds. Weighed liposomes or emulsion samples (100 mg) were dissolved in methanol and ethanol, respectively, by measuring the formation of fluorescence compounds resulting from interaction among lipid oxidation products and biological amino constituents (phospholipids). Fluorescence was measured at 345/416 nm (Perkin-Elmer LS 3B) and was standardized with a quinine sulfate solution (1 μg/mL in 0.05 M H_2SO_4) at the corresponding wavelengths (*33*). Duplicate analyses were performed for each sample.

Table 1. Phenolic Composition of Extra-Virgin Olive Oils (EVO) and Mill Wastewater[a]

compound	EVO1	EVO2	EVO3	mill wastewater
protocatechuic acid	0.9			
hydroxytyrosol (OHTy)	2.1	3.4	5.9	6.8
hydroxyphenylacetic acid	2.0			5.6
tyrosol (Ty)	11.5	6.4	22.2	16.6
caffeic acid	1.8			10.4
vanillic acid	2.4			7.2
p-coumaric acid	3.2			6.4
ferulic acid	1.1	5.1	2.7	0.2
o-coumaric acid	1.7	2.5	1.4	3.4
t-cinnamic acid	4.0	10.7	2.6	14.1
other simple phenols	13.0	20.0	22.4	23.1
dialdehydic form of elenolic acid linked with OHTy	8.8	5.0	8.6	
elenolic acid linked with Ty	2.2	20.7	9.2	
acetoxy-pinoresinol	6.0	7.2	7.4	
elenolic acid linked with OHTy	7.0	0.9	6.8	0.4
other polyphenols	10.5	12.4	5.6	5.4

[a] Quantitative data are given as percentage of total phenolics expressed as gallic acid, mean of triplicate analysis.

Statistical Analysis. The data were compared by one-way analysis of variance (ANOVA) (*34*), and the means were compared by a least squares difference method (*35*).

RESULTS

The effects of phenolic compounds on the antioxidant activity of lactoferrin were studied in liposomes and oil-in-water emulsions containing iron. In both lipid systems, lactoferrin and ferrous ion were compared at concentrations of 1:2 μM respectively, to reflect the lactoferrin capacity for binding two atoms of iron per molecule. In previous studies lactoferrin at 1 μM was shown to inhibit weakly the oxidation of liposomes containing 2 μM iron (*9*). However, this concentration of lactoferrin could not inhibit the oxidation of corn oil-in-water emulsions containing 2 μM iron.

Characterization of Phenol Composition of Extra-Virgin Olive Oils and Mill Wastewater. Phenol compositions of extra-virgin olive oils and mill wastewater are presented in **Table 1**. The most notable difference between olive oils and mill wastewater was the absence of complex phenols in mill wastewater. Tyrosol was the main compound in extra-virgin olive oil (EVO1 and EVO3) and in mill wastewater. Other simple compounds present in oils and mill wastewater were hydroxytyrosol, ferulic acid, cinnamic acid, and coumaric acid. Complex phenols extracted from oils were mainly concentrated in tyrosol- and hydroxytyrosol-derived compounds. The three olive oils were not very different in their total content of simple or complex phenols. However, their compositions showed some differences. EVO2 had a lower amount of ortho-diphenolic compounds and showed minor hydrolysis of secoridoid compounds as compared to EVO1 and EOV3. As for simple phenolics, EVO2 was higher concentrated in tyrosol and hydroxytyrosol than EOV1 and EOV3. The content of tocopherols, expressed as mg α-tocopherol/100 g oil, was the following: EVO1, 21.3 ± 0.2; EVO2, 9.8 ± 0.7; and EVO3, 14.2 ± 0.5.

Antioxidant Effects of Lactoferrin and α-Tocopherol. **Figure 1** shows the effects of different amounts of α-tocopherol added to liposomes containing lactoferrin and iron on the

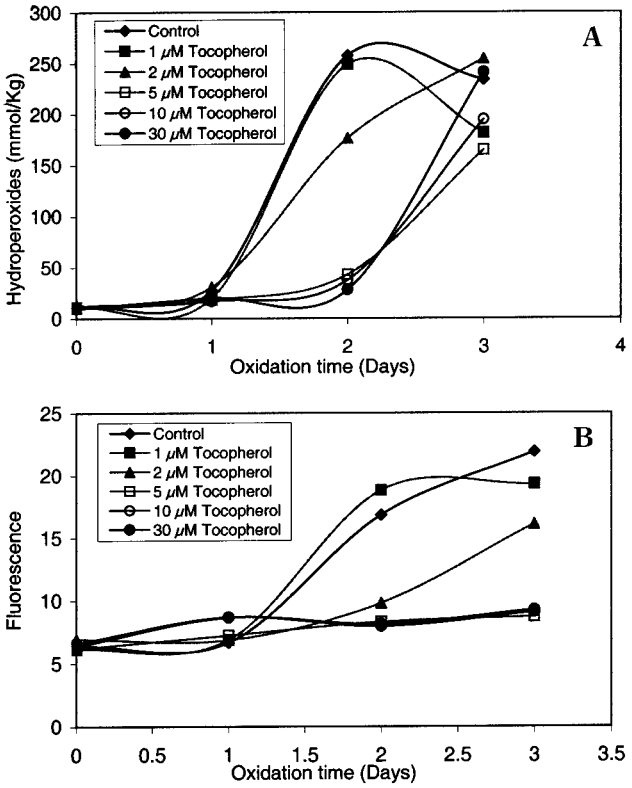

Figure 1. Effect of tocopherol concentration on the formation of hydroperoxides (A) and fluorescent compounds (B) in liposomes containing lactoferrin and iron (1:2 μM). Control contained lactoferrin and iron.

formation of hydroperoxides (A) and fluorescence compounds (B). α-Tocopherol added to lactoferrin retarded the formation of hydroperoxides and fluorescent compounds at concentrations higher than 1 μM. This inhibitory activity increased in the range between 2 and 5 μM α-tocopherol (**Table 2**). At concentrations of 10 and 30 μM, α-tocopherol showed similar inhibition as with 5 μM for the inhibition of both hydroperoxides and fluorescent oxidation compounds formation. The calculated synergism between 1 μM lactoferrin and 5 μM α-tocopherol was 5% by comparing the induction periods of formation of oxidation compounds [IP α-tocopherol, 18.6; IP lactoferrin, 4.9; IP (α-tocopherol + lactoferrin), 24.7].

The addition of 100 μM α-tocopherol to oil-in-water emulsions reinforced the iron binding activity of 1 μM lactoferrin in the presence of iron, resulting in inhibition of hydroperoxides and fluorescent formation at 30 °C (**Figure 2A** and **B**). Hydroperoxides formation was more inhibited at 500 than at 100 μM α-tocopherol, but inhibition did not increase at 1000 μM compared to that of 500 μM (**Table 2**). The inhibition of fluorescent formation increased with the α-tocopherol concentration. The synergistic effect between lactoferrin (1 μM) and α-tocopherol (500 μM) was calculated as 45% [IP α-tocopherol,: 6.2; IP lactoferrin, 5.2; IP (α-tocopherol + lactoferrin), 16.4].

Antioxidant Effects of Lactoferrin and Single Phenolic Compounds. Ferulic acid, tyrosol, and coumaric acid were tested for any cooperative effects with lactoferrin in liposomes and oil-in-water emulsions containing iron and compared with the effect of α-tocopherol. Ferulic acid, tyrosol, and coumaric acid at a concentration of 5 μM were highly effective in inhibiting the formation of hydroperoxides during oxidation of liposomes containing lactoferrin (**Figure 3A**). The order of inhibition was ferulic acid > α-tocopherol ≈ tyrosol > coumaric acid (**Table 3**). Ferulic acid was much more effective than tyrosol, coumaric acid, and α-tocopherol in inhibiting the formation of fluorescent compounds (**Figure 3B**). Therefore, ferulic acid showed the best cooperative effect with lactoferrin

Table 2. Inhibition by 1 μM Lactoferrin and Different Concentrations of α-Tocopherol on the Formation of Hydroperoxides and Fluorescent Compounds in Liposomes and Emulsions at 30 °C (mean ± SD)[a,b]

	% inhibition	
liposomes	hydroperoxides (day 2)	fluorescence (day 2)
control	0.0 ± 5.2[a]	0.2 ± 2.1[a]
+ 1 μM lactoferrin	2.5 ± 1.3[a]	2.1 ± 0.5[a]
+ 5 μM α-tocopherol	78.2 ± 2.2[c]	45.3 ± 1.6[b]
+ 1 μM lactoferrin + 1 μM α-tocopherol	3.4 ± 3.3[a]	−5.8 ± 2.0[a]
+ 1 μM lactoferrin + 2 μM α-tocopherol	31.5 ± 1.4[b]	41.7 ± 1.5[b]
+ 1 μM lactoferrin + 5 μM α-tocopherol	83.4 ± 0.6[d]	51.6 ± 0.8[c]
+ 1 μM lactoferrin + 10 μM α-tocopherol	85.4 ± 1.0[de]	52.6 ± 3.1[c]
+ 1 μM lactoferrin + 30 μM α-tocopherol	89.2 ± 0.1[e]	52.6 ±1.2[c]
	% inhibition	
emulsions	hydroperoxides (day 8)	fluorescence (day 8)
control	0.1 ± 3.4[a]	0.0 ± 0.5[a]
+ 1 μM lactoferrin	−5.1 ± 1.8[a]	2.4 ± 1.7[a]
+ 500 μM α-tocopherol	67.5 ± 1.3[c]	30.3 ± 2.4[b]
+ 1 μM lactoferrin + 100 μM α-tocopherol	39.0 ± 2.2[b]	31.3 ± 2.6[b]
+ 1 μM lactoferrin + 500 μM α-tocopherol	83.1 ± 1.9[d]	46.1 ± 4.1[c]
+ 1 μM lactoferrin + 1000 μM α-tocopherol	80.0 ± 5.0[d]	53.6 ± 2.1[d]

[a] Percent mean inhibition ± standard deviation of three samples; % inhibition = $[(C - S)/C] \times 100$, where C = oxidation product formed in control and S = oxidation product formed in sample; negative values represent prooxidant activity. [b] Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

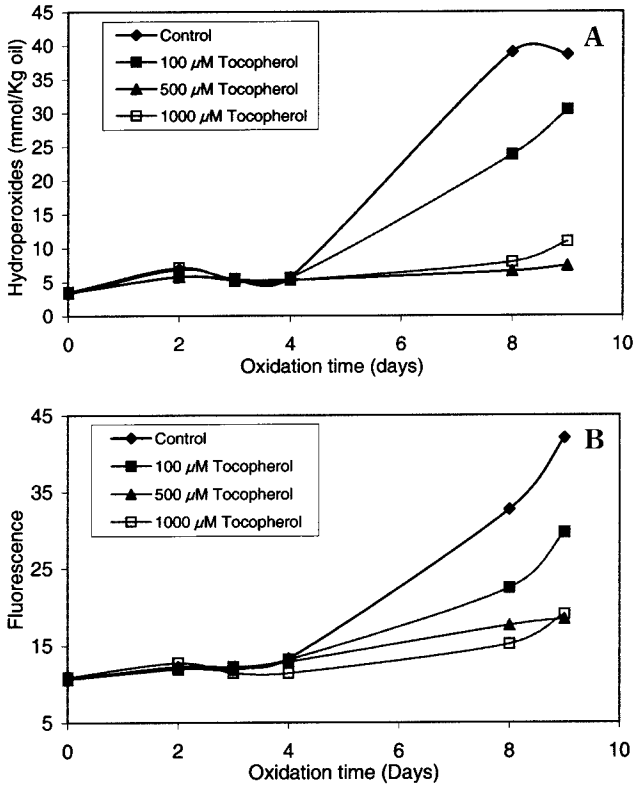

Figure 2. Effect of tocopherol concentration on the formation of hydroperoxides (A) and fluorescent compounds (B) in emulsions containing lactoferrin and iron (1:2 μM). Control contained lactoferrin and iron.

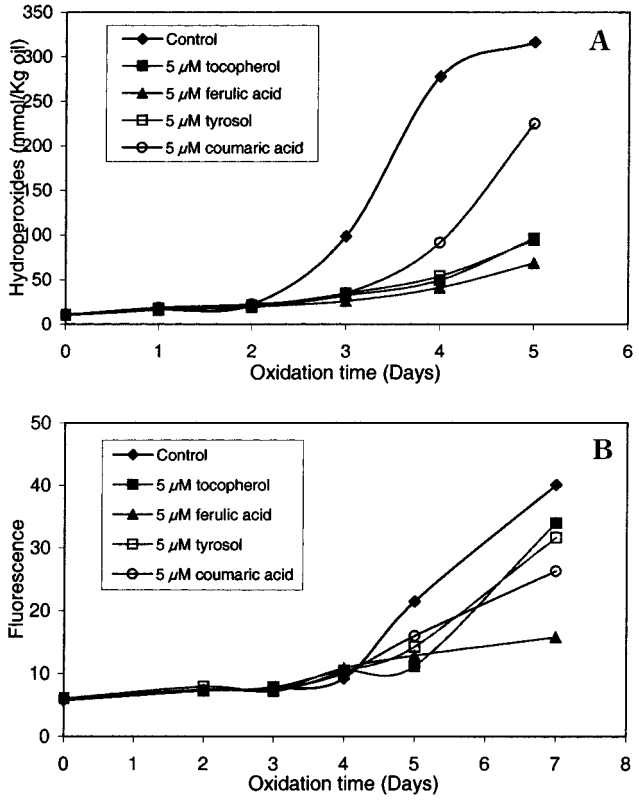

Figure 3. Effect of tocopherol and phenolic compounds on the formation of hydroperoxides (A) and fluorescent compounds (B) in liposomes containing lactoferrin and iron (1:2 μM). Control contained lactoferrin and iron.

in inhibiting the formation of hydroperoxides and fluorescence products. The synergism calculated between lactoferrin and ferulic acid for the oxidation compounds formation was 49% [IP ferulic acid, 6.1; IP lactoferrin, 4.9; IP (ferulic acid + lactoferrin), 16.3].

Ferulic acid and tyrosol also inhibited the oxidation of oil-in-water emulsions containing lactoferrin and iron at 30 °C (**Figure 4A** and **B**). The highest inhibition of hydroperoxide and fluorescence formation was observed at a concentration of 100−500 μM ferulic acid (**Table 3**). Ferulic acid at 500 μM inhibited fluorescent formation to the same extent as α-tocopherol at the same concentration. However, at concentrations higher than 500 μM, ferulic acid showed prooxidant activity by promoting the formation of fluorescent compounds. The best percent of inhibition was achieved with ferulic acid at 100 μM. The synergism calculated between lactoferrin 1 μM and ferulic acid at 100 μM on the formation of fluorescent in oil-in-water emulsions was only 6% [IP ferulic acid, 39.6; IP lactoferrin, 5.2; IP (ferulic acid + lactoferrin), 47.3] compared to 49% with the liposomes.

Tyrosol was less effective than ferulic acid, and more effective than α-tocopherol at the same concentration in inhibiting hydroperoxide formation. Tyrosol and ferulic acid at 100 μM, and α-tocopherol at 500 μM were equally effective in inhibiting the formation of fluorescent compounds (**Table 3**). The best inhibition of oxidation was achieved by tyrosol at 500 μM; higher concentrations did not increase the level of inhibition.

Antioxidant Effects of Lactoferrin and Phenolics Extracted from Olive Oils and Mill Wastewater. Because the optimal concentration of ferulic acid and α-tocopherol among those tested was 5 μM, phenolic extracts from the three extra-virgin olive oils and mill wastewater were tested at the same concentration in liposomes containing lactoferrin and iron. The addition of all phenolic extracts reduced hydroperoxide formation during oxidation of liposomes at 30 °C (**Figure 5A**). The inhibition was similar for all extracts obtained from extra-virgin olive oils and higher than for α-tocopherol and the phenolic extract of mill wastewater (**Table 4**). Ferulic acid showed similar inhibition as the extra-virgin olive oils extracts. Fluorescent formation was also inhibited to the same extent with all phenolic extracts in the presence of lactoferrin. The calculated synergistic effect between lactoferrin and phenolics extracted from extra-virgin olive oil (EVO1) was 126% [IP phenolic extract EVO1, 5.5; IP lactoferrin, 4.9; IP (phenolic extract EVO1 + lactoferrin), 23.5], and 47% for phenols extracted from mill wastewater [IP phenolic extract, 4.3; IP lactoferrin, 4.9; IP (phenolic extract + lactoferrin), 13.5].

In emulsions oxidized at 30 °C, all phenolic extracts of olive oils showed higher inhibition of hydroperoxide formation than ferulic acid at 100 μM (**Figure 6A**, **B**, **Table 4**). Ferulic acid and phenolic extracts were similar in inhibiting the formation of fluorescent compounds. No significant difference was observed between the antioxidant activity of phenolic compounds from mill wastewater and the three different extra-virgin olive oils tested. α-Tocopherol at 500 μM was less effective than phenolic compounds in inhibiting the formation of hydroperoxides and fluorescent compounds. The synergism calculated for phenolics extracted from extra-virgin olive oil and lactoferrin was 13% [IP phenolic extract EVO1, 28.2; IP lactoferrin, 5.2; IP (phenolic extract EVO1 + lactoferrin), 37.6], and 6% for phenolics extracted from mill wastewater [IP phenolic extract, 37.9; IP lactoferrin, 5.2; IP (phenolic extract + lactoferrin), 45.6].

Table 3. Inhibition by 1 μM Lactoferrin and Ferulic Acid, Tyrosol, and Coumaric Acid on the Formation of Hydroperoxides and Fluorescent Compounds in Liposomes and Emulsions at 30 °C (mean ± SD)[a,b]

	% inhibition	
liposomes	hydroperoxides (day 5)	fluorescence (day 7)
control	0.0 ± 0.1[a]	0.0 ± 0.6[a]
+ 1 μM lactoferrin	5.3 ± 0.9[b]	4.9 ± 1.2[b]
+ 5 μM ferulic acid	53.4 ± 1.1[d]	35.2 ±1.8[d]
+ 1 μM lactoferrin + 5 μM α-tocopherol	69.7 ± 0.3[e]	15.4 ± 3.0[c]
+ 1 μM lactoferrin + 5 μM ferulic acid	78.3 ± 3.5[f]	60.6 ± 4.9[e]
+ 1 μM lactoferrin + 5 μM tyrosol	70.3 ± 0.1[e]	21.2 ± 6.7[c]
+ 1 μM lactoferrin + 5 μM coumaric acid	29.0 ± 0.2[c]	34.4 ± 1.9[d]
	% inhibition	
emulsions	hydroperoxides (day 6)	fluorescence (day 6)
control	0.0 ± 0.9[b]	0.0 ± 2.2[b]
+ 1 μM lactoferrin	–10.5 ± 2.3[a]	1.8 ± 0.9[b]
+ 100 μM ferulic acid	71.2 ± 1.7[e]	20.8 ± 1.3[d]
+ 1 μM lactoferrin + 500 μM α-tocopherol	32.3 ± 3.1[c]	11.4 ± 6.1[c]
+ 1 μM lactoferrin + 100 μM ferulic acid	70.6 ± 4.8[e]	25.4 ± 7.6[df]
+ 1 μM lactoferrin + 500 μM ferulic acid	67.9 ± 1.1[e]	10.3 ± 3.5[c]
+ 1 μM lactoferrin + 1000 μM ferulic acid	59.9 ± 1.8[d]	–2.2 ± 3.3[b]
+ 1 μM lactoferrin + 2000 μM ferulic acid	63.1 ± 1.0[d]	–10.4 ± 2.3[a]
+ 1 μM lactoferrin + 100 μM tyrosol	30.2 ± 0.6[c]	18.2 ± 5.7[cd]
+ 1 μM lactoferrin + 500 μM tyrosol	61.4 ± 0.1[d]	26.8 ± 8.0[f]
+ 1 μM lactoferrin + 1000 μM tyrosol	73.1 ± 0.9[e]	28.8 ± 4.5[f]
+ 1 μM lactoferrin + 2000 μM tyrosol	71.5 ± 0.8[e]	24.7 ± 11.7[df]

[a] Percent mean inhibition ± standard deviation of three samples; % inhibition = $[(C - S)/C] \times 100$ where C = oxidation product formed in control and S = oxidation product formed in sample; negative values represent prooxidant activity. [b] Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

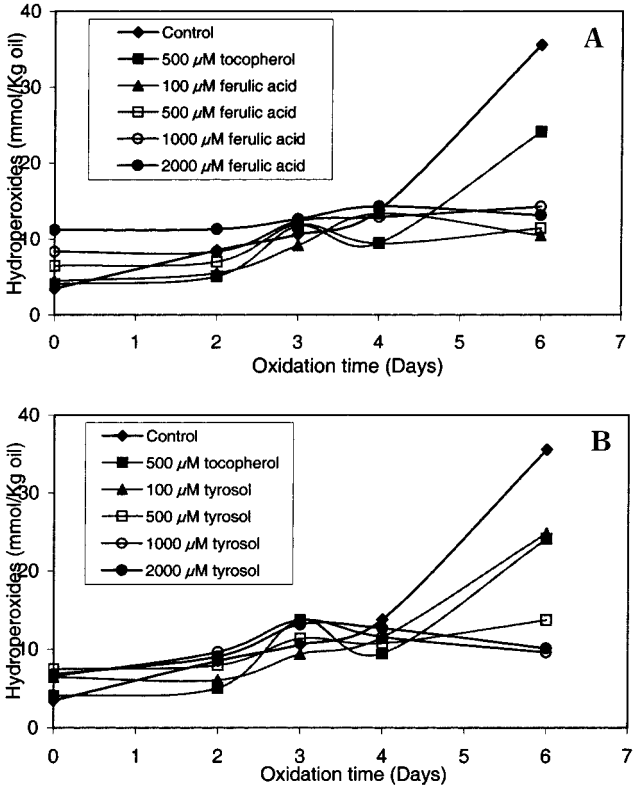

Figure 4. Effect of ferulic acid (A) and tyrosol (B) on the formation of hydroperoxides in emulsions containing lactoferrin and iron (1:2 μM). Control contained lactoferrin and iron.

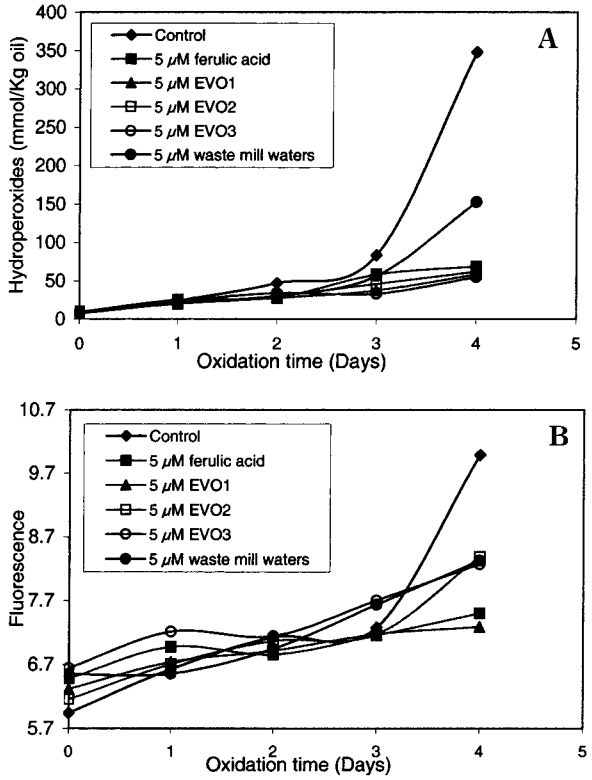

Figure 5. Effect of ferulic acid and phenolic compounds from extra-virgin olive oils and mill wastewater on the formation of hydroperoxides (A) and fluorescent compounds (B) in liposomes containing lactoferrin and iron (1:2 μM). Control contained lactoferrin and iron.

Effect of pH during Oxidation. Multicomponent food systems can undergo changes in pH during storage and processing by the accumulation of acid compounds, proteins, and phenolic compounds during oxidation. The effects of lactoferrin and phenolic compounds on the inhibition of hydroperoxides and fluorescent formation and on pH were studied in buffered liposomes and emulsions. A decrease of pH value was observed in liposomes after 9 days of oxidation (pH of control at day 0, 7.98, pH of control at day 11, 6.70), apparently due to the presence of acidic carbonyl compounds. In emulsions, lower pH differences were observed after 9 days of oxidation: (pH of control at day 0, 6.60, pH of control at day 11, 5.96). The pH of controls containing lactoferrin was not significantly different from the pH of samples containing lactoferrin and phenolic antioxidants during the entire incubation period. The synergistic effects between lactoferrin and phenolics in unbuffered systems were similar to those reported in buffered systems (data not shown).

Table 4. Inhibition by 1 μM Lactoferrin and Phenolic Extracts from Mill Wastewater and Extra-Virgin Olive Oils on the Formation of Hydroperoxides and Fluorescent Compounds in Liposomes and Emulsions at 30 °C (mean ± SD)[a,b]

	% inhibition	
liposomes	hydroperoxides (day 4)	fluorescence (day 4)
control	0.0 ± 2.3[a]	0.8 ± 1.7[a]
+ 1 μM lactoferrin	8.2 ± 1.5[b]	3.4 ± 0.7[a]
+ 5 μM EVO1	27.8 ± 3.0[c]	11.6 ± 1.3[b]
+ 5 μM mill wastewater	28.2 ± 2.7[c]	8.5 ± 2.1[b]
+ 1 μM lactoferrin + 5 μM α-tocopherol	69.7 ± 1.5[e]	25.3 ± 1.2[d]
+ 1 μM lactoferrin + 5 μM ferulic acid	80.3 ± 0.3[f]	24.9 ± 3.3[d]
+ 1 μM lactoferrin + 5 μM EVO1	83.2 ± 0.1[f]	27.0 ± 2.6[d]
+ 1 μM lactoferrin + 5 μM EVO2	82.2 ± 0.2[f]	16.0 ± 10.6[bcd]
+ 1 μM lactoferrin + 5 μM EVO3	84.3 ± 0.2[f]	17.2 ± 0.4[c]
+ 1 μM lactoferrin + 5 μM mill wastewater	56.2 ± 1.2[d]	16.6 ± 1.1[c]
	% inhibition	
emulsions	hydroperoxides (day 7)	fluorescence (day 7)
control	0.2 ± 4.8[a]	0.0 ± 2.1[b]
+ 1 μM lactoferrin	–5.6 ± 2.2[a]	–10.0 ± 3.2[a]
+ 100 μM EVO1	70.8 ± 1.4[d]	60.0 ± 3.5[e]
+ 100 μM mill wastewater	71.2 ± 2.5[d]	59.8 ± 2.8[e]
+ 1 μM lactoferrin + 500 μM α-tocopherol	11.0 ± 2.2[b]	11.4 ± 1.7[c]
+ 1 μM lactoferrin + 100 μM ferulic acid	36.1 ± 8.0[c]	51.0 ± 4.8[d]
+ 1 μM lactoferrin + 100 μM EVO1	77.4 ± 1.3[d]	55.1 ± 2.1[e]
+ 1 μM lactoferrin + 100 μM EVO2	75.3 ± 0.8[d]	56.7 ± 2.2[e]
+ 1 μM lactoferrin + 100 μM EVO3	79.1 ± 0.3[d]	57.5 ± 0.9[e]
+ 1 μM lactoferrin + 100 μM mill wastewater	73.4 ± 4.2[d]	54.7 ± 0.3[de]

[a] Percent mean inhibition ± standard deviation of three samples; % inhibition = $[(C - S)/C] \times 100$ where C = oxidation product formed in control and S = oxidation product formed in sample; negative values represent prooxidant activity. [b] Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

Effect of Bicarbonate. Bicarbonate has been reported to influence and stabilize the binding between lactoferrin and iron (*36*). However, previous reports indicated that the presence of bicarbonate did not modify the inhibition of hydroxyl radical generation by apo-lactoferrin at pH 7.4 (*37*). The addition of bicarbonate (2 μM) to liposomes containing a ratio of lactoferrin/iron of 1:2 slightly improved the antioxidant activity of lactoferrin (**Table 5**). The cooperative antioxidant effects between lactoferrin and phenolic compounds in inhibiting oxidation were not influenced by the presence of bicarbonate. The addition of bicarbonate improved the lactoferrin antioxidant activity in oil-in-water emulsions. However, the presence of bicarbonate slightly decreased the cooperative efforts between lactoferrin and phenolic compounds.

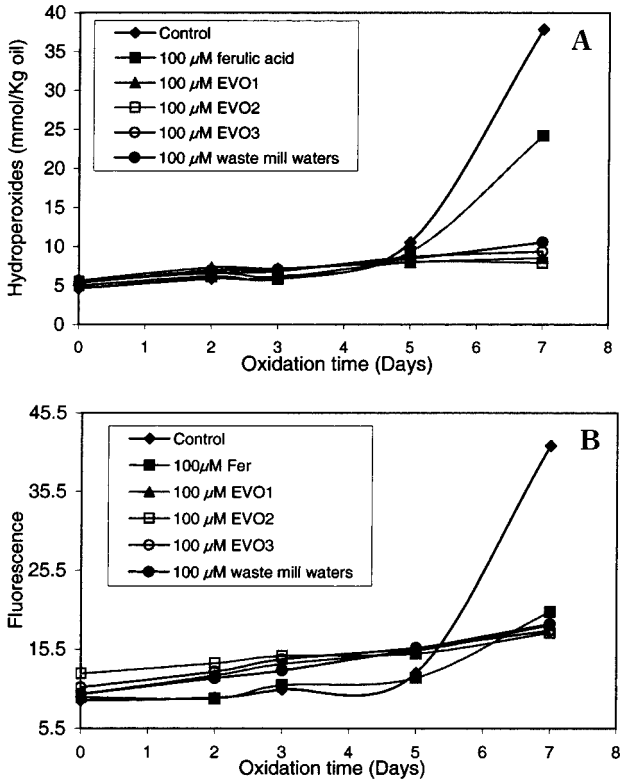

Figure 6. Effect of ferulic acid and phenolic compounds from extra-virgin olive oils and mill wastewater on the formation of hydroperoxides (A) and fluorescent compounds (B) in emulsions containing lactoferrin and iron (1:2 μM). Control contained lactoferrin and iron.

Table 5. Effect of Bicarbonate (HCO_3^-) 2 μM on the Inhibition by 1 μM Lactoferrin and Tocopherol and Phenolic Extracts from Extra-Virgin Olive Oil of the Formation of Fluorescent Compounds in Liposomes and Emulsions at 30 °C (mean ± SD)[a,b]

liposomes	% inhibition fluorescence (day 3)
control	0.0 ± 0.3[a]
control + $HC0_3^-$	0.1 ± 3.4[a]
+ 1 μM lactoferrin + $HC0_3^-$	12.9± 3.9[b]
1 μM lactoferrin + 5 μM α-tocopherol + $HC0_3^-$	24.2 ± 5.3[c]
1 μM lactoferrin + 5 μM EVO1 + $HC0_3^-$	73.5 ± 1.2[d]
emulsions	**% inhibition fluorescence (day 9)**
control	0.2 ± 0.2[a]
control + $HC0_3^-$	0.0 ± 5.1[a]
+ 1 μM lactoferrin + $HC0_3^-$	60.4 ±4.0[c]
1 μM lactoferrin + 500 μM α-tocopherol + $HC0_3^-$	45.9 ± 6.0[b]
1 μM lactoferrin + 100 μM EVO1 + $HC0_3^-$	49.0 ± 5.7[b]

[a] Percent mean inhibition ± standard deviation of three samples; % inhibition = $[(C - S)/C] \times 100$ where C = oxidation product formed in control and S = oxidation product formed in sample; negative values represent prooxidant activity. [b] Values in each column with the same superscript letter were not significantly different ($p < 0.01$).

DISCUSSION

The addition of lactoferrin was demonstrated to retard the oxidation of corn oil-in-water emulsions and liposomes containing metals, due to its metal-binding capacity (*9*). Lactoferrin can bind metal atoms, which become inactivated to catalyze lipid oxidation reactions, i.e., in the redox cycling and hydroperoxide decomposition reactions. However, when the concentration of iron exceeded lactoferrin's chelating ability, lactoferrin was unable to inhibit oxidation in lipid systems (*9*).

Several foodstuffs must be supplemented with relatively high amounts of iron for infant and aging populations. However, the presence of iron accelerates the rate of lipid oxidation and limits the shelf life of the product. In multicomponent systems containing lactoferrin, other compounds can reinforce its antioxidant capacity. All phenolic compounds tested in this study in liposomes and emulsion systems reinforced the antioxidant capacity of lactoferrin to different extents by additive or synergistic effects. The synergistic effects may be attributed to a protection of phenolics against oxidation by the presence of lactoferrin.

α-Tocopherol inhibited strongly the formation of hydroperoxides and fluorescent compounds in liposomes and emulsions. Lipophilic antioxidants such as α-tocopherol are recognized to be active antioxidants in protecting polar phospholipids in liposomes (*12*). Lipophilic antioxidants have also been shown to be effective in oil-in-water emulsions presumably by being oriented in the oil−water interface (*32*). α-Tocopherol showed an additive antioxidant effect with lactoferrin in the presence of iron in liposomes and it was a synergist of lactoferrin in emulsions, depending on the concentration. In both liposome and emulsion systems, the cooperative effect showed an optimum concentration for α-tocopherol, and higher concentrations did not improve the inhibition of hydroperoxide and fluorescent formation. Similarly, phenolic compounds lost their efficiency at high concentrations by regenerating peroxyl radicals (*12*).

The hydrophilic antioxidants, ferulic and coumaric acids and tyrosol, inhibited the formation of hydroperoxides and fluorescent compounds in both liposomes and emulsions. These antioxidants showed cooperative effects with lactoferrin, and ferulic acid was the best synergist in improving the antioxidant activity of lactoferrin. The methoxy group at the ortho position relative to the hydroxyl group in ferulic acid was reported to increase the antioxidant effectiveness by increasing their resonance stabilization and contributing to its chelating activity (*38*).

Ferulic acid showed a much higher synergism with lactoferrin in liposomes (49%) than α-tocopherol. Hydrophilic antioxidants have been described to have affinity for the polar surface of liposomal membranes being more effective than α-tocopherol that can remain inside the membrane (*12*). In liposomes the high synergism of ferulic acid may be attributed to the ability of lactoferrin to bind two atoms of iron and, thereby, protec phenolics against oxidation. However, in emulsions, lactoferrin and ferulic acid had only an additive inhibitory effect, which was lower than that observed between lactoferrin and α-tocopherol. The lower synergistic activity of ferulic acid in emulsions may be due to its partition to the aqueous phase where it may activate metals by reduction and antagonize any synergistic effects with lactoferrin.

All phenolic extracts resulting from extra-virgin olive oils and mill wastewater were effective antioxidants in retarding the formation of hydroperoxides and fluorescent compounds in liposomes and emulsions, which reinforced the effect of lactoferrin. In liposomes, phenolic extracts from olive oils were more effective than α-tocopherol, ferulic acid, and the extracts resulting from mill wastewater. In liposomes containing lactoferrin, phenol extracts from extra-virgin olive oils showed a strong synergism with lactoferrin (126%), which was higher than that observed with phenolics extracted from mill wastewater (47%) and ferulic acid (49%). In emulsion systems containing lactoferrin, phenolic extracts from oils and mill wastewater were

more effective in inhibiting oxidation than ferulic acid and α-tocopherol. These results indicated a higher antioxidant activity of lipophilic polyphenols extracted from extra-virgin olive oil than that of α-tocopherol and ferulic acid in emulsion systems. Fogliano et al. (*14*) have recently reported antioxidant activity of the dialdehydic form of elenolic acid linked with hydroxytyrosol occurred in extra-virgin olive oils as higher than that of free hydroxytyrosol (more abundant in mill wastewater) in micellar systems. The greater inhibition values obtained for phenolics extracted from mill wastewater than ferulic acid may be attributed to their composition with a significant content of caffeic acid. Caffeic acid is a more effective antioxidant than ferulic acid due to a second hydroxy group in the ortho position. Regarding the cooperative effects with lactoferrin, phenolic extracts from oils and mill wastewater used in combination with lactoferrin showed additive effects (13% and 6%, respectively) similar to those showed by ferulic acid. These lower values of synergism are probably related to the high antioxidant individual activity of phenolic extracts as described above.

Natural phenolic compounds were shown to reinforce the antioxidant capacity of lactoferrin to inhibit oxidation in lipid systems. Previous studies demonstrated the ability of lactoferrin to inhibit oxidation in foodstuffs such as infant formula (*21*, *39*). The present study showed that the antioxidant activity of lactoferrin can be improved by using phenolic natural antioxidants. This finding, in conjunction with the antimicrobial properties attributed to lactoferrin, makes the use of these ingredients as additives very advantageous in foods supplemented with iron.

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