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

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ABSTRACT

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 waste mill waters. The natural extracts of olive oils and waste-mill-waters were composed mainly of polyphenols and phenolic acids, respectively. Lipid oxidation at 30ºC 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, 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 multicomponents systems, antioxidants may reinforce each other by co-operative 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 metal chelating agent due to 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 attracted strong interest in their use as natural additives in foods (*13-15*), and they have demonstrated to have strong antioxidant activities (*16*)

Lactoferrin also shows bacteriostatic and bactericidal activities. These activities are

related with the iron deprivation of bacteria and the interaction of its N-terminal basic peptide with bacterial phospolipids membranes, respectively (*17,18*). The combined antioxidant and antimicrobial properties of lactoferrin have attracted strong interest and was demonstrated to be a useful supplement for some foods enriched in iron such as infant formula (*19*). This work was aimed at determining if 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 30ºC. 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: Lactoferrin from bovine, 90 % of purity, and 20% iron saturation, was obtained from Sigma (St. Louis, MO). Soybean lecithin (40% of L-α-phosphatidylcholine), potassium phosphate monobasic, potassium phosphate dibasic monohydrate, the Folin-Ciocalteu reagent, α -tocopherol (95% of 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 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). Commercial available corn oil was treated to remove natural tocopherols (*20*).

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

Preparation of phenolic extracts from oils and waste mill waters: The phenolic compounds from oils were extracted with ethanol and water (*21*). The phenolic compounds from waste mill waters were extracted using a previous acidification (pH: 2), a washing step with hexane and an extraction in ethyl acetate according to Brenes et al. (*22*). Total phenol content was determined by the Folin-Ciocalteu method (*23*) and expressed as gallic acid equivalents (GAE).

Phenol Composition by high-performance liquid chromatography (HPLC). Total

phenolic extracts were analysed by reverse-phase HPLC (*24*). 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 identified by Montedoro et al. (*25*) on the basis of their relative elution and ^{1}H-NMR spectroscopy (*24*).

Tocopherols. Tocopherols were analysed in extra virgin olive oils by reversed-phase HPLC (*26*).

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 (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 (10 mL) and bovine lactoferrin, ferrous sulfate and the different phenolic antioxidants were added.

Oxidation: Duplicate samples of liposomes and emulsions were oxidized with shaking at 30ºC. Oxidative stability was evaluated by measuring conjugated diene hydroperoxides and fluorescence compounds. Inhibition of oxidation was calculated during the propagation period of controls. Synergism was calculated by comparing the induction periods (IP) of formation of fluorescent compounds according to Frankel (*12*).

$$\%\ Sinergism = 100 \times \frac{[IP\ (Antiox._1 + Antiox._2) - (IP\ Antiox._1 + IP\ Antiox._2)]}{(IP\ Antiox._1 + IP\ Antiox._2)}$$

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 (UV-Vis Spectrophotometer Perkin Elmer,) and calculated as mmol hydroperoxydes/kg of oil as described previously (*27*).

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 of interaction among oxidation products and biological amino constituents (proteins, peptides, free amino acids and phospholipids). Fluorescence was measured at 345/416 nm (Perkin-Elmer LS 3B) and was standardised with a quinine sulphate solution (1 μg/mL in 0.05 M H_2SO_4) at the corresponding wavelengths (*28*).

Statistical Analysis. The data were compared by one-way analysis of variance (ANOVA) (*29*), and the means were compared by a least squares difference method (*30*).

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 waste-mill-waters: Phenol compositions of extra virgin olive oils and waste-mill-waters are presented in Table 1. The most difference between olive oils and waste-mill-waters was the absence of complex phenols in waste-mill-waters. Tyrosol was the main compound in extra virgin olive oil (EOV1 and EOV3) and in waste-mill-waters. Other simple compounds present in oils and waste-mill-waters were hydroxytyrosol, ferulic acid, cinnamic acid and coumaric acid. Complex phenols extracted from oils were mainly concentrated in tyrosol- and hydroxytyrosol- derived compounds. No significant differences were observed in the composition of the three olive oils regarding their content in simple or complex phenols.

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 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 20 μM, α-Tocopherol showed similar inhibition as with 5 μM for the inhibition of both hydroperoxides and fluorescence 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 30ºC (Fig. 2 A and B). Hydroperoxides formation was more inhibited at 500 than at 100 μM α-tocopherol, but did not increase 1000 μM compared to 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 acids 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 acids at a concentration of 5 μM were highly effective in inhibiting the formation of hydroperoxydes during oxidation of liposomes containing lactoferrin (Fig. 3 A). 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 (Fig

3 B). Therefore, ferulic acid showed the best cooperative effect with lactoferrin 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 30ºC. (Figures 4 A and B). The highest inhibition of hydroperoxide and fluorescence formation was observed at 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 waste-mill waters: To obtain an optimal concentration of ferulic acid and α-tocopherol calculated as 5 μM, phenolic extracts from the three extra virgin olive oils and waste-mill

waters 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 30ºC (Fig 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 waste-mill-waters (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 (EOV1) was 126% [IP phenolic extract EOV1: 5.5; IP lactoferrin: 4.9; IP (phenolic extract EOV1 + lactoferrin): 23.5], and 47% for phenols extracted from waste-mill-waters [IP phenolic extract: 4.3; IP lactoferrin: 4.9; IP (phenolic extract + lactoferrin): 13.5].

In emulsions oxidized at 30ºC, all phenolic extracts of olive oils showed higher inhibition of hydroperoxide formation than ferulic acid at 100 μM (Fig. 6 A, 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 waste-mill-waters 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 EOV1: 28.2; IP lactoferrin: 5.2; IP (phenolic extract EOV1 + lactoferrin): 37.6], and 6% for phenolics extracted from waste-mill-waters [IP phenolic extract: 37.9; IP lactoferrin: 5.2; IP (phenolic extract + lactoferrin): 45.6].

Effect of pH during oxidation: Multicomponent food systems can undergo pH

changes 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 were studied in liposomes and emulsion prepared in water. A slight decrease of pH value was observed in liposomes after nine 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 than the pH of samples containing lactoferrin and phenolic antioxidants during the entire incubation period. The synergistic effects between lactoferrin and phenolics were similar in unbuffered systems as those reported in buffered systems (data not shown).

Effect of bicarbonate*:* Bicarbonate has been reported to influence and stabilise the binding between lactoferrin and iron (*31*). However, previous reports indicated that the presence of bicarbonate did not modify the inhibition of hydroxyl radical generation of apo-lactoferrin at pH 7.4 (*32*). 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 co-operative 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 co-operative efforts between lactoferrin and phenolic compounds.

DISCUSSION

The addition of lactoferrin was demonstrated to retard the oxidation of corn oil-in-water emulsions and liposomes containing metals, due 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 multicomponents 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. Lipophylic antioxidants such as α-tocopherol are recognised to be active antioxidants in protecting polar phospholipids in liposomes (*12*). Lipophylic antioxidants have also been shown to be effective in oil-in water emulsions presumably by being oriented in the oil-water interface (*27*). α-Tocopherol showed an additive antioxidant effect with lactoferrin in the presence of iron in liposomes and it was synergist of lactoferrin in emulsions, depending on the concentration. In both liposome and emulsion systems, the co-operative 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 hydrophylic antioxidants, ferulic and coumaric acids and tyrosol inhibited both, the formation of hydroperoxides and fluorescent compounds, in liposomes and emulsions. These antioxidants showed co-operative 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 stabilisation and contributing to its chelating activity (*33*).

Ferulic acid showed a much higher synergism with lactoferrin in liposomes (49%) than α-tocopherol. Hydrophylic 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 for binding two atoms of iron and thereby, protecting 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 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 waste mill waters 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 waste mill waters. 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 waste-mill waters (47 %) and ferulic acid (49 %). In emulsion systems containing lactoferrin, phenolic extracts from oils and waste mill waters were more effective in inhibiting oxidation than ferulic acid and α-tocopherol. These results indicated a higher antioxidant activity of lypophylic polyphenols extracted from extra virgin olive oil than α-tocopherol and ferulic acid in emulsion systems. The greater inhibition values obtained for phenolics extracted from waste-mill waters than ferulic acid may be attributed to their composition with a significant content in caffeic acid. Caffeic acid is a more effective antioxidant than ferulic acid due to a second hydroxy group in the *orto* position. Regarding the co-operative effects with lactoferrin, phenolic extracts from oils and waste-mill waters used in combination with lactoferrin showed similar additive effects (13 % and 6 %, respectively) as those showed by ferulic acid. These lower values of synergism are probably related with 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 infant formula (*19*,*34*). 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 very advantageous as additives in foods supplemented with iron.

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Table 1. Phenolic Composition of Extra Virgin Olive Oils (EOV) and Waste-mill-waters [a]

Compound	EOV1	EOV2	EOV3	Waste—mill-waters
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
Siryngic acid	---	---	---	---
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 and expressed as caffeic acid, mean of triplicate analysis.

Table 2. Inhibition by 1 μM Lactoferrin and Different Concentrations of α-Tocopherol on the Formation of Hydroperoxydes and Fluorescent Compounds in Liposomes and Emulsions at 30ºC (mean±sd) [1,2].

Liposomes	Hydroperoxides (day-2) (% Inhibition)	Fluorescence (day-2) (% Inhibition)
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]
Emulsions	**Hydroperoxides (day-8) (% Inhibition)**	**Fluorescence (day-8) (% Inhibition)**
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]

Table 3. Inhibition by 1 μM Lactoferrin and Ferulic acid, Tyrosol and Coumaric acid on the Formation of Hydroperoxydes and Fluorescent Compounds in Liposomes and Emulsions at 30ºC (mean±sd) [1,2].

Liposomes	Hydroperoxides (day-5)	Fluorescence (day-7)
	(% Inhibition)	
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}
Emulsions	Hydroperoxides (day-6)	Fluorescence (day-6)
	(% Inhibition)	
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}

Table 4. Inhibition by 1 μM Lactoferrin and Phenolic Extracts from Waste-mill-waters and Extra Virgin Olive Oils on the Formation of Hydroperoxydes and Fluorescent Compounds in Liposomes and Emulsions at 30ºC (mean±sd)[1,2].

Liposomes	Hydroperoxides (day-4) (% Inhibition)	Fluorescence (day-4) (% Inhibition)
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 EOV1	27.8 ± 3.0[c]	11.6 ± 1.3[b]
+ 5 μM waste mill waters	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 EOV1	83.2 ± 0.1[f]	27.0 ± 2.6[d]
+ 1 μM lactoferrin + 5 μM EOV2	82.2 ± 0.2[f]	16.0 ± 10.6[bcd]
+ 1 μM lactoferrin + 5 μM EOV3	84.3 ± 0.2[f]	17.2 ± 0.4[c]
+ 1 μM lactoferrin + 5 μM waste mill waters	56.2 ± 1.2[d]	16.6 ± 1.1[c]
Emulsions	**Hydroperoxides (day-7) (% Inhibition)**	**Fluorescence (day-7) (% Inhibition)**
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 EOV1	70.8 ± 1.4[d]	60.0 ± 3.5[e]
+ 100 μM waste mill waters	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 EOV1	77.4 ± 1.3^{d}	55.1 ± 2.1^{e}
+ 1 μM lactoferrin + 100 μM EOV2	75.3 ± 0.8^{d}	56.7 ± 2.2^{e}
+ 1 μM lactoferrin + 100 μM EOV3	79.1 ± 0.3^{d}	57.5 ± 0.9^{e}
+ 1 μM lactoferrin + 100 μM waste mill waters	73.4 ± 4.2^{d}	54.7 ± 0.3^{de}

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Table 5. Effect of Bicarbonate (CO_3^{2-}) 2 μM on the Inhibition by 1 μM Lactoferrin and Tocopherol and Phenolic Extracts from Extra Virgin Olive Oil on the Formation of Fluorescent Compounds in Liposomes and Emulsions at 30ºC (mean±sd)[1,2].

Liposomes	Fluorescence (day-3) (% Inhibition)
Control	0.0 ± 0.3
Control + $C0_3^{2-}$	0.1 ± 3.4[a]
+ 1 μM lactoferrin + $C0_3^{2-}$	12.9± 3.9[b]
1 μM lactoferrin + 5 μM α-tocopherol + $C0_3^{2-}$	24.2 ± 5.3[c]
1 μM lactoferrin + 5 μM AOJ + $C0_3^{2-}$	73.5 ± 1.2[d]
Emulsions	**Fluorescence (day-9) (% Inhibition)**
Control	0.2 ± 0.2
Control + $C0_3^{2-}$	0.0 ± 5.1[a]
+ 1 μM lactoferrin + $C0_3^{2-}$	60.4 ±4,0[b]
1 μM lactoferrin + 500 μM α-tocopherol + $C0_3^{2-}$	45.9 ± 6.0[b]
1 μM lactoferrin + 100 μM AOJ + $C0_3^{2-}$	49.0 ± 5.7[c]

FIGURE LEGENDS

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.

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

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 waste mill waters on the formation of hydroperoxides (A) and fluorescent compounds (B) in liposomes containing lactoferrin and iron (1:2 μM). Control contained lactoferrin and iron.

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