Comparative effect of bovine buttermilk, whey, and lactoferrin on the innate immunity receptors and oxidative status of intestinal epithelial cells

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Abstract

Milk contains active molecules with important functional properties as the defensive proteins; among them are the whey protein lactoferrin and proteins of the milk fat globule membrane (MFGM) present in buttermilk. The aim of this study has been to investigate the effect of lactoferrin, whey and buttermilk as modulators of intestinal innate immunity and oxidative stress on intestinal epithelial cells, to evaluate its potential use for the development of functional foods. Innate immune Toll-like receptors (TLR2, TLR4 and TLR9) mRNA expression, lipid peroxidation (MDA+4-HDA) and protein carbonyl levels were analyzed in enterocyte-like Caco-2/TC7 cells treated for 24 hours with different concentrations of lactoferrin, whey or buttermilk. None of the substances analyzed caused oxidative damage; however, whey significantly decreased the levels of lipid peroxidation. Furthermore, both lactoferrin and whey were able to reduce the oxidative stress induced by lipopolysaccharide. Respect to TLR receptors, lactoferrin, whey and buttermilk specifically altered the expression of TLR2, TLR4 and TLR9 receptors, with a strong decrease in TLR4 expression. These results suggest that lactoferrin, whey and buttermilk could be interesting potential ingredients for functional foods as they seem to modulate oxidative stress and inflammatory response induced by TLRs activation.

Keywords: intestine, oxidative stress, TLR2, TLR4, TLR9

Introduction

Bovine milk is the most consumed type of milk, and several dairy products have also been produced and consumed worldwide for millennia. Evidence from recent studies, systematic reviews, and meta-analyses indicates that milk and dairy products may reduce or modify the risk of developing cardiometabolic diseases, overweight, obesity and type 2 diabetes mellitus, and probably protect against specific types of cancer, such as colorectal cancer (Thorning et al. 2016; Fontecha et al. 2019; Hidayat et al. 2019). Milk, as well as whey and buttermilk, two important by-products of dairy industry, contain active molecules characterized by having functional properties (Svanborg et al. 2015). Among them are the defensive proteins, such as the whey protein lactoferrin, and some proteins located in the milk fat globule membrane (MFGM).

Whey is obtained after casein coagulation in cheese manufacture. Traditionally considered a waste product, whey proteins are currently being used to improve technological properties of foods or as an ingredient in functional foods, due to their outstanding functional and nutritional properties (de Wit 1998). Buttermilk, a dairy by-product released in the butter-making process, has been also undervalued for many years. However, several studies have shown that it contains high value-added components, especially the proteins and polar lipids present in the MFGM (Vanderghem et al. 2010).

Lactoferrin is a glycoprotein present in many exocrine secretions of mammals, such as milk, saliva and tears (Siqueiros-Cendón et al. 2014), with the ability to bind two ferric atoms per molecule (Baker and Baker 2004). Lactoferrin develops an antimicrobial function by sequestering iron and depriving bacteria from it (Orsi 2004, Jenssen and Hancock 2009), and by altering the permeability of the bacterial outer membrane (Orsi 2004). Lactoferrin exerts antiviral activity by blocking the virus surface or by hampering their binding to the cellular receptors (Van der Strate et al. 2001). Lactoferrin is also involved in some immune processes, such as inhibition of inflammation or promotion of innate and adaptive immune responses (Legrand et al. 2005; Actor et al. 2009), decreasing the production of reactive oxygen species and modulating the pro-inflammatory cytokines (Kruzel et al. 2017).

In the digestive tract, the intestinal epithelium is a critical barrier between the body and intestinal content, which activates innate immunity responses to maintain its integrity and physiology (Cario 2008). Toll-like receptors (TLRs) play a key role in the innate immune system, recognizing microbial-associated molecular patterns (MAMPs) and contributing to inflammatory responses (Fukata et al. 2009). In this context, TLR2 and TLR4 recognize cellular components of Gram-positive and Gram-negative bacteria, respectively, while TLR9 detects specific bacterial DNA motifs. The intestinal epithelium is also a major target for oxidative damage due to the constant exposure of reactive oxygen species (ROS) generated by luminal contents (Gill et al. 2010).

Therefore, the aim of the present work has been to determine and compare the effect of whey, buttermilk and lactoferrin on mRNA expression of several TLRs, and on the oxidative stress of lipids and proteins in Caco-2/TC7 cells, as a model of human intestinal epithelia.

Materials and methods

Cell culture

This study was carried out in the human cell line Caco-2/TC7, since these cells are an excellent human enterocyte-like model to study intestinal epithelial physiology (Mesonero et al. 1994). The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2, and maintained in high-glucose DMEM, supplemented with 2% L-glutamine (2 mM), 1% penicillin (10,000 U/mL) and streptomycin (10 mg/mL), 1% nonessential amino acids (10 mM), and 20 % fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, USA). The cells were passaged enzymatically (0.25% trypsin-1 mM EDTA), subcultured in 25 cm^2 cell culture flasks (Sarstedt, Nuembrecht, Germany), and seeded at a density of 10^4 cells/cm^2. The experiments were carried out in cells cultured for 15 days until morphological and functional enterocyte-like differentiation. The medium was changed 72 h after seeding the cells and then every 48 h. For the experiments, the cells were seeded in 6-well plates at a density of 2×10^5 cells/well. Cells were treated for 24 h with FBS-free culture medium containing either different concentrations of lactoferrin, whey, buttermilk, and/or lipopolysaccharide (LPS) (3 μg/mL) (*E. coli* 0111:B4;

Sigma-Aldrich, St Louis, MO, USA). The samples were analyzed in triplicate in at least three independent experiments.

Lactoferrin, whey and buttermilk preparation

Bovine lactoferrin was kindly donated by Tatua Nutritionals Company (Morrinsville, New Zealand). The purity of lactoferrin was checked by SDS-PAGE, which showed a single band corresponding to a protein of about 80 kDa and purity higher than 90%. Lactoferrin had an iron-saturation below 10%. A concentrated solution of lactoferrin was prepared in ultrapure water and it was filtered through a low-binding protein 0.22 µm filter. After filtration, the absorbance of lactoferrin solutions was measured at 280 nm and its concentration determined by considering an extinction coefficient of 12.7 (280 nm, 1% solution).

The procedure for obtaining buttermilk and whey from raw bovine milk was performed as previously described (Parrón et al. 2017). Briefly, raw cow milk provided by the dairy industry Villacorona (El Burgo de Ebro, Zaragoza, Spain) was skimmed in a cream separator (ARR-DES 125, Suministros Químicos Arroyo, Santander, Spain). The cream was stored at 4 °C overnight and afterwards was churned into butter, obtaining the buttermilk released in the process that was filtered through cheesecloth and stored at -20 °C until use. Skimmed milk was fractionated into casein and whey by casein coagulation at 35 °C for 45 min with recombinant chymosin (Chr. Hansen, Hørsholm, Denmark). The whey fraction was recovered by decanting and filtering through glass wool, and it was stored at -20 °C until use. Concentrated solutions of whey and buttermilk were dissolved in ultrapure water and filtered through low-binding protein 0.22 µm filters before the experiments with cells.

The concentration of lactoferrin in whey and buttermilk was determined by radial immunodiffusion according to Sánchez et al. (1992). The content of LPS in lactoferrin was determined by the colorimetric kit ToxinSensorTM Chromogenic LAL (GenScript, New York, USA).

Cell homogenate preparation

For cell homogenate preparation, the cells were resuspended and homogenized with cold Tris-mannitol buffer (200 mM Tris, 500 mM mannitol, a protease inhibitor cocktail (Complete Mini, EDTA-free; Roche, Barcelona, Spain), 2% sodium azide, 25 mg/mL benzamidine, and 100 mM phenylmethylsulfonyl fluoride (PMSF)), pH 7.1. Then, the homogenate was disrupted by sonication (15 1-s bursts, 60 W) and centrifuged for 10 min at 4 °C and 3,000 *g*, and the supernatant was taken for lipid peroxidation and protein carbonyl analysis (Latorre et al. 2014b).

Measurement of lipid and protein peroxidation

Caco-2/TC7 cells were treated with lactoferrin, whey or buttermilk at 0.5, 1, 2, 5 or 10 mg/mL concentration during 24 h. Lipid and protein peroxidation were assayed as previously described (Latorre et al. 2014b). Therefore, the level of lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA). Briefly, MDA + 4-HDA reacted with N-methyl-2-phenyl-indole and yielded a stable chromophore that was measured in a spectrophotometer at 586 nm, using 1,1,3,3-tetramethoxypropane as standard. The results were calculated in nmol of MDA + 4-HDA/mg protein and were expressed as the percentage of the control value (100%). Protein oxidation was analyzed by carbonyl level measurement. Cell homogenates were incubated with the classical carbonyl reagent 2,4-dinitrophenylhydrazine (DNPH), and protein carbonylation was measured spectrophotometrically at 375 nm. The results were calculated in nmol carbonyl groups/mg protein and were expressed as percentage of the control value (100%). Protein content was measured by following the Bradford method (Bio-Rad, Hercules, CA, USA).

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from cells cultured in 6-well plates (15 days after seeding) as previously described (Latorre et al. 2018), using the RNeasy mini kit and the RNase-free DNase set from Qiagen and following the manufacturer's instructions. The extracted RNA (1 μg) was used as a template for first-strand cDNA synthesis using oligo (dT) primers and a reverse

transcriptase (qScript cDNA SuperMix - Quanta Biosciences Inc., Gaithersburg, MD, USA). cDNA obtained by reverse transcription (RT) were used to determine TLR2, TLR4 and TLR9 mRNA expression levels by StepOne Plus Real-Time PCR System (Life Technologies, Paisley, UK), using SYBR Green, with GAPDH and HPRT1 as housekeeping genes. The specific primers used are detailed in Table 1. Each sample was run in triplicate, and the mean Ct was determined from the three runs. Relative mRNA expression under each experimental condition (control or treatment) was expressed as $\Delta Ct = Ct_{gene} - Ct_{calibrator}$. Then, relative mRNA expression was calculated as $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$. Finally, the relative gene expression levels were converted and expressed as fold difference ($=2^{-\Delta\Delta Ct}$).

Statistical analysis

The results are expressed as mean ± standard error (SE) of the mean. Statistical comparisons were performed using one-way ANOVA followed by the Dunnett post-test with a confidence interval of 95 % ($p < 0.05$), and normal distribution was previously confirmed with the D'Agostino–Pearson test. Statistical analysis is indicated in each figure and was developed with the computer-assisted Prism GraphPad Program (Prism version 7.0, GraphPad Software, San Diego, CA, USA).

Results

Effect of lactoferrin, whey and buttermilk on lipid and protein oxidation

The results show that Caco-2/TC7 cells treated with different concentrations of lactoferrin or buttermilk display a slight increase in the oxidation of proteins, while treatment with whey shows a small decrease; however, none of these differences were significant at any of the concentrations assayed, compared with the control (Figure 1A, 1B, and 1C).

Regarding to lipid peroxidation, whey treatment of Caco-2/TC7 cells show a significant reduction in the oxidation levels at all tested concentrations except at the lowest (0.5 mg/mL) (Figure 1E). Conversely, but in agreement to previous results, lactoferrin and buttermilk did not appear to modify the lipid oxidative status of cells in a significant way (Figure 1D and 1F).

The concentration of lactoferrin found in milk fractions was of 0.91 mg/g of lyophilized whey and of 0.48 mg/g of lyophilized buttermilk.

Effect of lactoferrin, whey and buttermilk on TLR2, TLR4, and TLR9 mRNA expression

Since lactoferrin has been involved in processes as inhibition of inflammation or promotion of innate and adaptive immune responses (Legrand et al. 2005; Puddu et al. 2011), we analyzed if treatment with lactoferrin, whey or buttermilk could alter TLR2, TLR4 and TLR9 mRNA expression in Caco-2/TC7 cells differentiated into enterocyte-type cells. As shown in Figure 2, lactoferrin, whey and buttermilk altered the expression of TLR2, TLR4 and TLR9 receptors in a different and specific way.

None of the treatment of cells with lactoferrin and buttermilk seemed to affect TLR2 mRNA expression. However, the expression of this receptor was significantly increased by the highest concentration of whey treatment (10 mg/mL) (Figure 2A).

Analysis of TLR4 mRNA expression level revealed that it was significantly diminished, at the concentration of 10 mg/mL of lactoferrin, whey and buttermilk (Figure 2B).

Finally, the results obtained show that the concentration of 0.5 mg/mL of buttermilk induces a significant increase in TLR9 expression. This increase was also observed in cells treated with lactoferrin, although it was not statistically significant. Oppositely, the highest concentration of buttermilk tested (10 mg/mL) induced a significant decrease in TLR9 mRNA expression (Figure 2C).

Effect of lactoferrin, whey and buttermilk on oxidative stress after LPS challenge

Oxidative stress level was measured on basal state of the cells, i.e. in a situation of normal homeostatic balance. In addition, lactoferrin, whey and buttermilk strongly decreased the expression of TLR4, mainly at high concentrations. Therefore, we decided to analyze the effect of lactoferrin, whey and buttermilk on cells previously stimulated with LPS, a major component of the outer membrane of Gram-negative bacteria recognized by TLR4, which we have previously shown to increase the oxidation of both proteins and lipids (Latorre et al. 2014b).

206 Then, the cells were treated with LPS (3 μg/mL) and/or lactoferrin, whey and buttermilk (0.5
207 and 10 mg/mL) throughout the course of 24 h. Interestingly, the results show that the two tested
208 concentrations of whey and lactoferrin were able to reduced lipid protein oxidation induced by
209 LPS (Figure 3B). Oxidative damage in proteins yielded by LPS was reverted by lactoferrin at
210 the highest concentration assayed (10 mg/mL) and by the two concentrations of whey (0.5 and
211 10 mg/mL) (Figure 3A). In contrast, buttermilk was not able to inhibit or reduce the oxidative
212 stress produced by LPS treatment.

213 The concentration of LPS in the 10 mg/mL lactoferrin solution was of 8×10^{-6} μg/mL.

214 **Discussion**

215 Biological systems are normally subjected to a certain level of oxidative stress; however, when
216 reactive oxygen species exceed certain quantities, some severe physiological disorders can
217 occur (Park et al. 2017). Consequently, there is great interest in finding natural substances that
218 can decrease cellular oxidative stress, and thus some vegetal compounds and milk proteins have
219 been investigated for this purpose. For instance, whey proteins have been proposed as
220 antioxidants in several foods to replace chemical antioxidants (Hu et al. 2003; Peña-Ramos and
221 Xiong 2003; Giblin et al. 2019). Therefore, in this study we have investigated in Caco-2/TC7,
222 the effect of buttermilk, whey and lactoferrin, on their basal oxidative stress and after being
223 challenged with LPS. We have also evaluated the effect of those compounds on the expression
224 of Toll-like receptors 2, 4 and 9.

225 The results obtained showed that the treatment of Caco-2/TC7 cells with whey reduced their
226 basal oxidative stress, although the reduction was only significant for lipid peroxidation.
227 Interestingly, our results agree with those obtained by Piccolomini et al. (2012) that evaluated
228 the protective effect of whey products on ROS levels in Caco-2 cells subjected to H_2O_2. It was
229 shown that whey protein isolate and hydrolysate products, at concentrations of 1 and 2 mg/mL,
230 caused a significant reduction in ROS levels. Another study carried out on the cell line PC12 of
231 rat adrenal phaeochromocytoma (Jin et al. 2013) demonstrated that whey protein hydrolysates
232 protected those cells against H_2O_2-induced oxidative stress, by reducing apoptosis and

233 increasing antioxidant enzyme activities. The extensive review by Corrochano et al. (2018)
234 includes several studies about the activity of whey and whey proteins as antioxidants, as well as
235 the effect of heat treatments and hydrolysis on such activity. They concluded that although
236 studies reveal that direct cell exposure to whey samples increases intracellular antioxidants,
237 such as glutathione; however, *in vivo* studies are not yet completely conclusive.

238 The addition of buttermilk or lactoferrin to Caco-2/TC7 cells did not produce any significant
239 effect on the oxidative stress of proteins and lipids. In the case of buttermilk, our results
240 disagree with some previous studies that reported antioxidant activity of this by-product
241 (Conway et al. 2013; Ripollés et al. 2016). However, it has to be considered that in those studies
242 the antioxidant activity was not evaluated on a cellular model, but by chemical assays. On the
243 other hand, one study carried out on the human epithelial colon cancer cell line HT-29 showed
244 that apo-lactoferrin and lactoferrin saturated with selenium were able to regulate the altered
245 redox state of those cells, by exerting antioxidant activity (Burrow et al. 2011). Those authors
246 suggested that lactoferrin could be a valuable component as chemopreventive agent or as a
247 supplement to improve the immunological level. However, our results are not coincident with
248 those of Burrow et al. (2011); this discrepancy could be explained by the different cell line used
249 in both studies.
250 In a model of Caco-2 cells subjected to hydrogen peroxide to mimic the damage produced in
251 processes with intestinal epithelial injury (Liu et al., 2019), the addition of human recombinant
252 lactoferrin down-regulated some inflammatory markers and up-regulated intestinal stem cells
253 and epithelial proliferation markers. Moreover, lactoferrin was also shown to increase the
254 expression of tight junction proteins and the transepithelial electrical resistance in Caco-2 cells,
255 along with a decrease in paracellular permeability (Zhao et al. 2019).

256 Intestinal epithelium is a major target for oxidative damage due to constant exposure of reactive
257 oxygen species (ROS) generated by luminal contents, including microbiota (Gill et al. 2010). In
258 this sense, oxidative stress and activation of TLR pathways can mutually promote each other. In
259 fact, ionizing radiation-induced ROS can increase TLR2 and TLR4 expression through de novo

protein synthesis pathway (Yoshino and Kashiwakura 2017), while activation of TLR2, TLR3 and TLR4 can enhance the oxidative status of intestinal epithelial cells (Latorre et al. 2014b).

In this work, we have also observed that the expression of the innate immune receptors TLR2, TLR4, and TLR9, which expression has been demonstrated in Caco-2 cells after certain stimulus, such as bacteria, virus or specific agonists (Wang et al. 2012; 2008; Latorre et al. 2018; Hiramatsu et al. 2019), was modified by whey, buttermilk and lactoferrin, although in a different way each of them. Our results show that lactoferrin, whey and buttermilk diminish TLR4 expression, however, only whey seems to increase TLR2 expression and only buttermilk might affect TLR9 expression. These results agree, in part, with those obtained in previous studies, in which the effect of lactoferrin and milk fractions were evaluated on the TLRs of monocytes and peripheral blood mononuclear cells, respectively (Puddu et al. 2011; Kiewiet et al. 2017).

Our results show that lactoferrin, whey and buttermilk strongly decrease the expression of TLR4. In this sense, previous studies have shown that lactoferrin interacts with antigen presenting cells, thus having an effect on the expression of soluble immune mediators, such as cytokines, thus regulating inflammation and immunity (Legrand et al. 2005; Puddu et al. 2009). In fact, it has been demonstrated that blocking TLR2 and TLR4 in monocytes affect the induction of IL-6 mediated by bovine lactoferrin, which indicates a critical role of lactoferrin in directing host immune function (Puddu et al. 2011).

As far as TLR2 is concerned, our results showed no effect of lactoferrin or buttermilk; however, an increase in TLR2 expression was observed in cells treated with high concentrations of whey. Interestingly, TLR2 has been shown to contribute to epithelial barrier function by different mechanisms, including the organization of tight junction zonula occludens 1 protein (ZO-1) (Cario et al. 2004) and the increase of intestinal mucosa repair and renewal (Hörmann et al. 2014), being postulated to ameliorate intestinal injury induced by chronic inflammatory processes.

It is of great interest that our results show the opposite effect on the modified expression of TLR4 and TLR9, decreasing the levels of the former and increasing those of the latter. In other studies, the effect of bovine lactoferrin on several TLRs on human monocytic leukemia cell line has been explored, showing a TLR4 activation (Figueroa-Lozano et al. 2018). On the other hand, it has been reported that TLR9 plays a role in one of the major pathways responsible for the anti-inflammatory effects of genomic DNA from lactobacilli (Kim et al. 2012), and that TLR9 in epithelial cells can neutralize inflammatory signals induced by the activation of other TLRs (Vijay 2018). Therefore, an increase in TLR9 expression, as induced by lactoferrin and whey could help in the anti-inflammatory effects, even though the effects of lactoferrin have not been statistically significant.

Except for the whey that reduces lipid peroxidation, our results do not show a significant effect of lactoferrin, whey or buttermilk on the basal state of cellular oxidation. However, both lactoferrin and whey have shown an ability to reverse LPS-induced oxidative damage in lipids and proteins. In the case of lactoferrin, this effect can be exerted by direct binding to LPS, as it has been proved that lactoferrin neutralizes free LPS, by avoiding the formation of LPS complexes that activate the TLR4 signaling pathways (Drago-Serrano et al. 2012). In some studies performed *in vivo*, Kruzel et al. (2010) showed that lactoferrin attenuated mitochondrial dysfunction in LPS-treated animals, suggesting a protective role for lactoferrin in mitochondrial ROS production (Kruzel et al. 2010).

The protective effect of lactoferrin on the action of LPS has been studied in *in vitro* and *in vivo* models. Thus, Hirotani et al. (2008) showed in Caco-2 cells that LPS decreased their barrier permeability, altering the expression of two key tight junction proteins. The authors also proved an increase of oxidative damage by LPS, which was attenuated by lactoferrin from human milk. In the study by Kruzel et al. (2002) provoked a LPS-induced endotoxaemia in mice and administered intraperitoneally human lactoferrin as a prophylactic, concurrent or therapeutic event relative to endotoxic shock. It was found that lactoferrin exerted a differential regulation of pro-inflammatory and anti-inflammatory mediators.

The content of lactoferrin in the whey and buttermilk fractions used in this study (4.8 and 9.1 μg/ml, respectively, in the most concentrated samples) is very low compared to the assayed concentrations of lactoferrin. Therefore, the effect of whey and buttermilk observed could be attributed to the rest of proteins and peptides present, as they are a complex mixture. The different effect of whey, compared to buttermilk, in the reversion of LPS-induced oxidative damage in lipids and proteins could be related with the presence in whey of glycomacropeptide (GMP). This compound is released from kappa-casein by the enzymatic action of chymosin when casein is coagulated in cheese manufacture. In some studies GMP, and specially its hydrolysates, have been proposed to have a modulatory activity on macrophage TLR4 and potential LPS inhibitors (Cheng et al. 2015). These results suggest that the effects of lactoferrin and whey may be evident in tissues that have been exposed to inflammatory or oxidative stress promoting conditions, as previous studies have concluded for some anti-inflammatory cytokines such as IL-10 (Latorre et al. 2014a). Moreover, the greater antioxidant effect observed for whey, in part, may be due to the combination of a strong reduction in TLR4 along with an increase in TLR2 and TLR9 expression. Thus, a lower expression of TLR4 would add to the beneficial effects described for TLR2 (Cario et al. 2004; Hörmann et al. 2014) and TLR9 (Kim et al. 2012; Vijay 2018).

Taking into account that there are few studies on the activity of milk proteins on oxidative stress and TLR expression, and since our results show some contradictory effects depending on the TLR analyzed, it should be necessary to carry out further experiments to wide our knowledge on the influence of dairy fractions and proteins on the innate immune system, as well as the inflammatory responses of tissues, such as the intestine.

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Table 1. Primer sequences used for real-time PCR analysis of expression of Toll-like receptor and housekeeping genes in Caco-2/TC7 cells.

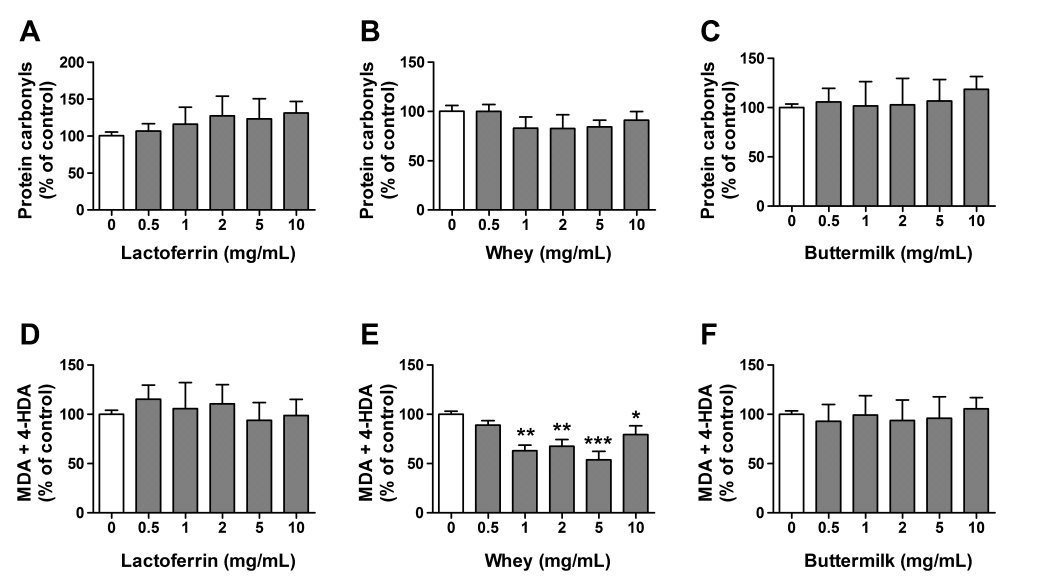
Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
TLR2	GAAAGCTCCCAGCAGGAACATC	GAATGAAGTCCCGCTTATGAAGACA	146
TLR4	TTGAGCAGGTCTAGGGTGATTGAAC	ATGCGGGACACACACACTTTCAAATA	143
TLR9	AGTCCTCGACCTGGCAGGAA	GCGTTGGCGCTAAGGTTGA	168
HPRT1	CTGACCTGCTGGATTACA	GCGACCTTGACCATCTTT	256
GAPDH	CATGACCACAGTCCATGCCATCACT	TGAGGTCCACCACCCTGTTGCTGTA	137

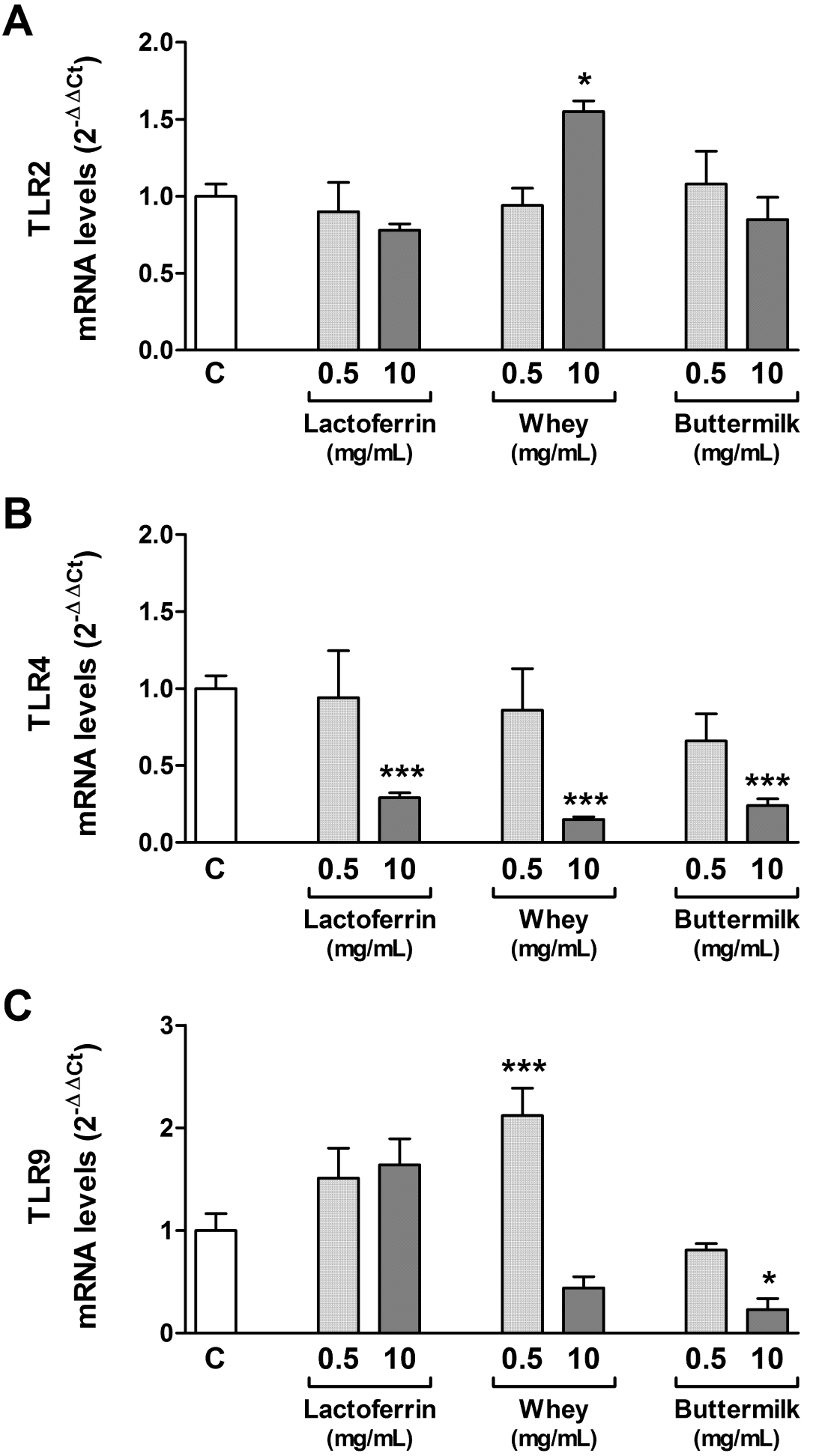
Figure captions

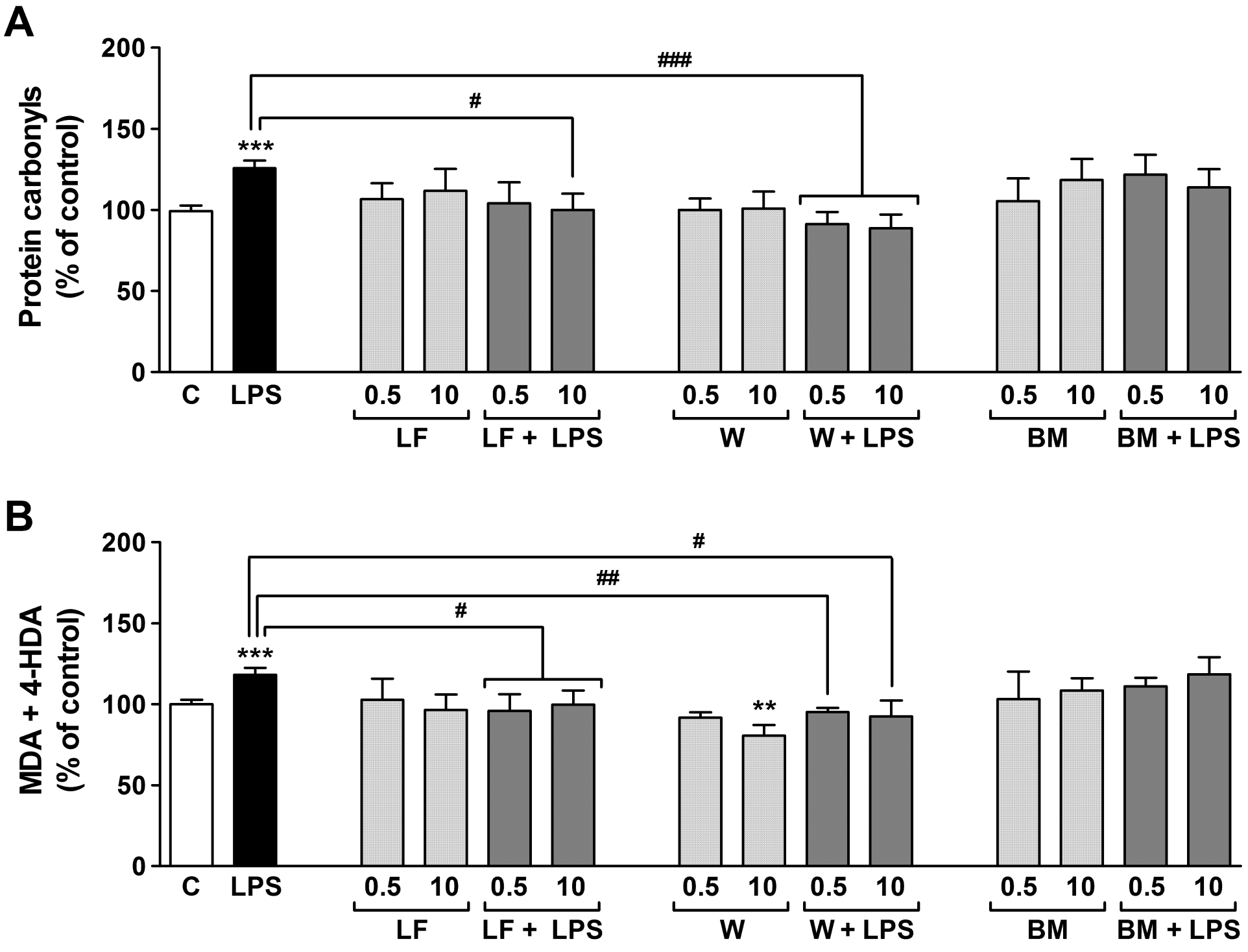
Fig, 1. Effect of lactoferrin, whey and buttermilk on protein oxidation (A-C) and lipid peroxidation (D-E). Caco-2/TC7 cells were treated during 1 day with lactoferrin, whey or buttermilk at 0.5, 1, 2, 5 or 10 mg/mL. Results were expressed as the percentage of the control value (100%) and were indicated as the mean ± SE of three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, compared with control.

Fig. 2. Real-time PCR analysis of TLR2, TLR4 and TLR9 mRNA expression: modulation by lactoferrin, whey and buttermilk treatment. Caco-2/TC7 cells were treated with lactoferrin, whey or buttermilk at either 0.5 or 10 mg/mL concentration during 1 day. Relative quantification of mRNA expression was performed using comparative Ct method ($2^{-\Delta\Delta Ct}$). Results are expressed as arbitrary units (control = 1) and are the mean ± SE of three independent experiments. *$p < 0.05$ and ***$p < 0.001$ compared with the corresponding control.

Fig. 3. Effect of lactoferrin (LF), whey (W), buttermilk (BM) and/or LPS on protein oxidation (A) and lipid peroxidation (B). Caco-2/TC7 cells were treated during 1 day with either LPS 3 µg/mL and/or lactoferrin (LF), whey (W) or buttermilk (BM) at two concentrations, 0.5 and 10 mg/mL. Results were expressed as the percentage of the control value (100%) and were indicated as the mean ± SE of four independent experiments. **$p < 0.01$ and ***$p < 0.001$, compared with control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with LPS treatment.


A
Protein carbonyls (% of control)
200
150
100
50
0
0 0.5 1 2 5 10
Lactoferrin (mg/mL)
B
Protein carbonyls (% of control)
150
100
50
0
0 0.5 1 2 5 10
Whey (mg/mL)
C
Protein carbonyls (% of control)
150
100
50
0
0 0.5 1 2 5 10
Buttermilk (mg/mL)
D
MDA + 4-HDA (% of control)
150
100
50
0
0 0.5 1 2 5 10
Lactoferrin (mg/mL)
E
MDA + 4-HDA (% of control)
150
100
50
0
**
**
***
*
0 0.5 1 2 5 10
Whey (mg/mL)
F
MDA + 4-HDA (% of control)
150
100
50
0
0 0.5 1 2 5 10
Buttermilk (mg/mL)


A
TLR2
mRNA levels ($2^{-\Delta\Delta Ct}$)
2.0
1.5
1.0
0.5
0.0
*
C
0.5
10
0.5
10
0.5
10
Lactoferrin (mg/mL)
Whey (mg/mL)
Buttermilk (mg/mL)
B
TLR4
mRNA levels ($2^{-\Delta\Delta Ct}$)
2.0
1.5
1.0
0.5
0.0
***
***
***
C
0.5
10
0.5
10
0.5
10
Lactoferrin (mg/mL)
Whey (mg/mL)
Buttermilk (mg/mL)
C
TLR9
mRNA levels ($2^{-\Delta\Delta Ct}$)
3
2
1
0
***
*
C
0.5
10
0.5
10
0.5
10
Lactoferrin (mg/mL)
Whey (mg/mL)
Buttermilk (mg/mL)


A
Protein carbonyls (% of control)
200
150
100
50
0
###
#
***
C
LPS
0.5
10
0.5
10
0.5
10
0.5
10
0.5
10
0.5
10
LF
LF + LPS
W
W + LPS
BM
BM + LPS
B
MDA + 4-HDA (% of control)
200
150
100
50
0
#
##
#
***
**
C
LPS
0.5
10
0.5
10
0.5
10
0.5
10
0.5
10
0.5
10
LF
LF + LPS
W
W + LPS
BM
BM + LPS