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Protective effect of bovine lactoferrin against *Cronobacter sakazakii* in human intestinal Caco-2/TC7 cells

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ABSTRACT

Milk is a source of bioactive proteins with defensive properties of great value for protecting the newborn. The activity of bovine milk lactoferrin (LF) was investigated as an antibacterial agent in the internalisation of the emergent pathogen *Cronobacter sakazakii* into Caco-2/TC7 cells, a model of human intestinal epithelium. The effect of LF on oxidative stress and expression of Toll-like receptors (TLR) was also investigated. LF exerted a clear antibacterial activity against *C. sakazakii*, as well as an inhibitory effect on *C. sakazakii* adhesion and internalisation into Caco-2/TC7 cells. Incubation with *C. sakazakii* induced an increase in oxidative stress on the lipid fraction of Caco-2/TC7 cells, which was reversed by LF. Additionally, LF altered the expression of TLR, with a clear decrease in the expression levels of TLR4 at 24 h of incubation. These results suggest that LF has very interesting properties as a potential ingredient for functional foods.

1. Introduction

Milk from mammals is a source of bioactive proteins, some of them with defensive properties, which are of great value for protecting the newborn (Svanborg, Johansen, Abrahamsen, & Skeie, 2015). The dairy industry generates a large volume of by-products that implicate an environmental problem. The current destination of those dairy by-products is mainly to obtain technological ingredients or to become supplements for animal feeding. The isolation of bioactive components from these by-products would add an extra value to them.

In 2016, the global generation rate of whey was estimated at 200 MT per year, with an annual increase of 3% (Domingos et al., 2018). Whey obtained in cheese-manufacture accounts for 95% of the world's liquid whey; the remaining 5% derives from industrial isolation of caseins, which has decreased in the last few years. Whey contains more than 50% of milk solids, and includes lactose, whey proteins, minerals and vitamins. Its composition depends on the procedure of casein coagulation (De Wit, 1998). Whey proteins can be defined as the milk proteins that remain soluble after precipitation of caseins by acidification or enzymatic coagulation (Madureira, Pereira, Gomes, Pintado, & Malcata, 2007).

The main whey proteins are β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, lactoferrin and lactoperoxidase, among others. Some of them are associated with antimicrobial and antiviral activities, immune system stimulation, anticarcinogenic activity and other metabolic functions (Madureira et al., 2007). Lactoferrin (LF), one of the milk defensive proteins, is present in many exocrine secretions of mammals, such as milk, tears and saliva (Siqueiros-Cendón et al., 2014). LF is a cationic glycoprotein from the transferrin family, with iron-binding capacity and numerous properties (García-Montoya, Cendón, Arévalo-Gallegos, & Rascón-Cruz, 2012), including antibacterial activity against Gram-positive and Gram-negative bacteria. LF is secreted in its open form without iron (apo-LF) and binds two ferric ions (Fe^{3+}) to give rise to its closed form (holo-LF) (Mayeur, Spahis, Pouliot, & Levy, 2016). The most recognised activity of LF is bacteriostatic when it does not have iron, because of its ability to sequester iron

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from the environment (Jenssen & Hancock, 2009). However, the iron-saturated LF can also be active by interacting with the bacterial membrane by destabilising it (González-Chávez, Arévalo-Gallegos, & Rascón-Cruz, 2009) and, consequently, altering the metabolism of bacteria.

LF is one of the most important elements for the defence against infections and the proper development and maturation of the intestinal mucosa (Aly, Ros, & Frontela, 2013). LF is also involved in some immunological processes, as it can inhibit inflammation or promote the innate and adaptive responses of the immune system (Actor, Hwang, & Kruzel, 2009). This activity of LF causes a decrease in reactive oxygen species (ROS) production and modulates the pro-inflammatory cytokines (Kruzel, Zimecki, & Actor, 2017).

During the last few decades, *Cronobacter sakazakii* has appeared as an emerging pathogen, causing great concern because it has been mainly associated with the consumption of infant formula and milk powder. Furthermore, this pathogen has also been isolated from various environments and food products (Iversen & Forsythe, 2003). *C. sakazakii* mainly affects the intestinal and digestive system, being especially dangerous for infants and neonates, to whom it can cause other severe symptoms (Iversen & Forsythe, 2003) and diseases like meningitis, necrotising enterocolitis or sepsis (Quintero et al., 2011).

C. sakazakii belongs to the Enterobacteriaceae family and is a Gram-negative motile bacillus, non-spore-forming and facultative anaerobic. It is capable of growing in a wide temperature range, from 6 to 47 °C, its optimum temperature being 39 °C (Iversen & Forsythe, 2003). *C. sakazakii* also has high resistance to low water activities, in the range from 0.3 to 0.83. All these properties give this pathogen the ability to grow in a great variety of foods (Lin & Beuchat, 2007).

The infection mechanism of *C. sakazakii*, after oral intake of contaminated foods, is based on the epithelial cell damage to cross the intestinal barrier, thus reaching the circulatory system (Ribet & Cossart, 2015). This pathogen interacts with intestinal epithelial cells, causing intestinal inflammation and villus alterations. *C. sakazakii* requires cellular structures to multiply and invade the intestine (Kim et al., 2010), stage that is considered critical to the disease pathogenesis (Giri et al., 2012). There are some evidences supporting that the addition of natural antimicrobials, such as LF, can avoid the growth of *C. sakazakii* in reconstituted powdered infant formula (Harouna et al., 2020). It has been reported that the glycans present in milk, similar to the carbohydrates of the cell surface, interfere with the adhesion of bacteria to cells by binding to pathogens (Newburg, Ruiz-Palacios, & Morrow, 2005). Therefore, if milk glycans can bind to receptors located on bacterial membrane, they could also bind to specific regions of the cellular surface avoiding bacterial adhesion (O'Riordan, Kane, Joshi, & Hickey, 2014).

Infection with pathogenic agents can cause oxidative stress in the cells that are affected by them (Ivanov, Bartosch, & Isaguliants, 2017). Therefore, there is great interest to find natural substances that can avoid or decrease cellular stress, which is caused when ROS exceed certain levels, producing physiological disorders (Park et al., 2017). An important target for oxidative damage is the intestinal epithelium, as it is subjected to constant exposure to ROS due to luminal content and microbiota, even in the intestine not affected by pathogens (Gill, Tsung, & Billiar, 2010).

Innate immunity plays a key role in inflammation by acting as the primary host defence system to respond quickly to microbial attack and tissue damage (De Nardo, 2015). Among the components of immune system that initiate the innate immune response are Toll-like receptors (TLRs), which act recognising pathogen-associated molecular patterns (PAMPs). When TLRs detect PAMPs, they undergo a conformational change that promotes the activation of intracellular signalling pathways, triggering an intestinal response, and stimulating the synthesis of pro-inflammatory cytokines or interferon alpha or beta (Layunta, Buey, Mesonero, & Latorre, 2021). In this context, TLR2 recognises a wide variety of PAMPs, such as lipopeptides, peptidoglycan and lipoteichoic acid, present in the cell wall of Gram-positive bacteria. TLR2 has been reported to improve the epithelial barrier function through different mechanisms, including the organisation of the tight junction zonula occludens-1 protein. TLR4 recognises cellular components of bacteria, such as the lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria, having a protective effect in gut injuries, and TLR9 recognises intracellular bacteria (Burgueño & Abreu, 2020).

Therefore, the main aim of this study was to evaluate bovine LF as an antimicrobial agent against the emergent pathogen *C. sakazakii*, in the cellular line Caco-2/TC7 differentiated in enterocytes, used as a model of human intestinal epithelium. In addition, the activity of this protein on oxidative stress and expression of some intestinal Toll-like receptors in Caco-2/TC7 cells infected with *C. sakazakii* were analysed.

2. Material and methods

2.1. Preparation of lactoferrin

Bovine LF used for all assays was kindly donated by Tatua Nutritionals (Morrinsville, New Zealand). LF had an iron-saturation below 10% and a purity higher than 90%, which was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), following the procedure described by Laemmli (1970), and showing a single band corresponding to a protein of about 80 kDa. Furthermore, a double immunodiffusion was performed to confirm that commercial LF was free of lactoperoxidase using specific rabbit polyclonal antibodies obtained previously in our laboratory, following the procedure described by Navarro, Harouna, Calvo, Pérez, and Sánchez (2015). These procedures were approved by the Ethic Committee for Animal Experiments of the University of Zaragoza (Project Licence PI48/10). The care and use of animals were performed as stated in the Spanish Policy for Animal Protection RD 53/2013, which meets the EU Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

In addition, the presence of LPS in commercial LF was determined using the ToxinSensor™ Chromogenic LAL colorimetric kit (GenScript, NJ, USA) for the detection of bacterial endotoxins, according to manufacturer's instructions. In brief, the sample was incubated for 9 min at 37 °C with LAL reagent and the change in absorbance at 545 nm was measured. Kruzel et al. (2013) used the same kit and procedure to assess endotoxin levels present in human recombinant LF.

For the assays, a concentrated stock solution of LF was prepared in ultrapure water and sterilised using a low-binding protein filter of 0.22 μm. LF concentration was determined after filtration from its molecular extinction coefficient ($E^{1\%}_{280} = 1.27\ mL\ cm^{-1}\ g^{-1}$). The concentration of LF solutions used in the different assays was adjusted considering the concentration of the stock solution.

2.2. Culture conditions of C. sakazakii

The bacterial strain used in this study was *C. sakazakii* CECT 858, supplied by the Spanish Type Culture Collection (CECT, Valencia, Spain), which corresponds with the strain ATCC 29544 of the American Type Culture Collection. This strain is of clinical origin from a child throat and is recommended as reference strain by international standards ISO 22964:2017 (ISO, 2017) and ISO

11133:2014/Amd 2:2020 (ISO, 2020). For the reference stock, the bacteria were fixed to porous rings and stored in cryovials at −80 °C. To cultivate *C. sakazakii*, a porous ring was transferred to a tube with 10 mL of triptycase soy broth (TSB) (Merck, Darmstad, Germany) supplemented with 0.6% (w/v) yeast extract (YE) (Oxoid, Basingstoke, UK) and incubated for 24 h at 37 °C in aerobiosis conditions. Afterwards, the culture was seeded by depletion on a plate of trypticase soy agar (TSA) (Merck) supplemented with 0.6% (w/v) YE and incubated at 37 °C for 24 h to isolate the colonies for the assays.

2.3. Cell culture

This study was carried out in the human cell line Caco-2, clone TC7. Several authors have used this cell line to study intestinal epithelial physiology because it is an excellent model of human enterocyte-like cells (Latorre, Mendoza, Layunta, Alcalde, & Mesonero, 2014; Mesonero et al., 1994; Vašíček et al., 2020). It was maintained with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% (v/v) heat-inactivated foetal bovine serum, 1% (v/v) penicillin (10,000 units mL^{-1}) -streptomycin (10 mg mL^{-1}), 1% (v/v) non-essential amino acids (NEAA) (100 ×) and 1% (v/v) L-glutamine (200 mM) (all reagents from Biological Industries, Kibbutz Beit Haemek, Israel). The culture flasks were maintained at 37 °C in a saturated humid atmosphere with 95% air and 5% CO_2. A solution of 0.25% (w/v) trypsin with 1 mmol L^{-1} EDTA (ThermoFisher Scientific, Rockford, IL, USA) was used to enzymatically passage the cells, which were subcultured in 25 cm^2 cell culture flasks (Sigma–Aldrich, St. Louis, MO, USA) at a density of 10^4 cells per cm^2. The experiments were carried out with cells cultured for 15 days to obtain a differentiation stage and morphology similar to functional enterocytes (Mesonero et al., 1994). The medium was changed every 48 h. For the assays with LF, the medium used was basic DMEM, supplemented with 1% (v/v) L-glutamine and 1% (v/v) NEAA, to avoid interactions with foetal bovine serum.

2.4. Bioactivity assays

2.4.1. Antibacterial activity of lactoferrin against C. sakazakii

This assay was conducted using *C. sakazakii* culture obtained from exponential and stationary phases, to check whether the effect of LF on bacterial viability depended on the growth phase.

A single colony of *C. sakazakii*, isolated as described above, was incubated in 10 mL TSB with 0.6% (w/v) YE for 8 h (exponential phase) or for 18–20 h (stationary phase) at 37 °C in aerobiosis. Then, serial dilutions of the bacterial suspension were made with 1% (w/v) peptone water to reach a 4–5 log cfu mL^{-1} suspension that was added to a 96-well microtitre plate (100 μL per well). Samples of native LF, at different concentrations (0.5, 1, 2, 5 and 10 mg mL^{-1}), were mixed with the bacterial suspension at a 1:1 ratio (v/v). All samples were assayed in duplicate in three independent experiments. A control, only with bacterial suspension, was included in each experiment. The plate was maintained at 37 °C in aerobiosis and an aliquot of each sample was extracted at 4 and 24 h of incubation, subjected to decimal dilutions in peptone water and seeded on TSA plates. The colonies were counted after 24 h of incubation of the plates at 37 °C.

2.4.2. Effect of lactoferrin on Caco-2/TC7 cell viability

The effect of LF on viability of Caco-2/TC7 cells was evaluated before the inhibition of bacterial internalisation assays. First, 96-well plates were seeded with Caco-2/TC7 cells at a density of 5×10^3 cells per well and maintained in culture for 15 days to achieve their differentiation into enterocytes. Cells were incubated with LF solutions dissolved in basic DMEM at different concentrations (0.5, 1, 2, 5 and 10 mg mL^{-1}), each in triplicate. In this assay, the effect of LF was tested incubating the cells with this protein at two different times (1 and 24 h) in three independent experiments.

Differentiated cells were washed with 200 μL of sterile PBS. Afterwards, 200 μL per well of basic medium were added to the cells for them to get used to this simple medium and the plate was incubated for 2 h at 37 °C. Afterwards, the cells were washed and 50 μL of LF at different concentrations were added per well. A solution of 50 mM H_2O_2 was used as positive control for cytotoxicity and basic DMEM as negative control. The plate was incubated for 1 h at 37 °C.

In the second type of assay, the cells were previously incubated with different concentrations of LF at 37 °C for 24 h, to assess whether a longer incubation with LF had any effect on the cell response. Then, the assay was completed with the process detailed above.

The evaluation of the viability of the cells, after incubation with the samples, was carried out using the CellTiter 96 AQueous One Solution Cell Proliferation kit (Promega, Madison, WI, USA). The cells were washed and 200 μL per well of basic medium and 20 μL of 3-(4, 5-dimethyl thiazol-2-yl)-5-(3-carboxy methoxy phenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) reagent were added. MTS allows the determination of the number of viable cells, as this reagent is bioreduced in the formazan dye by the activation of NADPH dependent dehydrogenase enzymes of metabolically active cells. The formazan is soluble in the culture medium, and after 2 h of incubation at 37 °C, the absorbance can be read at 492 nm on a Multiskan MS ELISA plate reader (Labsystem, Helsinki, Finland) and is directly proportional to the number of viable cells.

2.4.3. Inhibition of C. sakazakii internalisation by Caco-2/TC7 cells

The objective of this assay was to evaluate if LF was able to inhibit the internalisation of *C. sakazakii* into Caco-2/TC7 cells by incubating them with different concentrations of the protein before the contact with bacteria.

This assay was carried out in 24-well plates cultured with cells at a density of 3×10^4 cells per well for 15 days. The effect of LF was tested in three independent experiments at two different times (1 and 24 h). The only difference between both assays was that in the second case, the cells were previously incubated with different concentrations of LF for 24 h, as it has been explained in the viability assay.

Differentiated cells were washed with 2 mL per well of sterile PBS and left for 2 h at 37 °C with basic DMEM. Afterwards, each well was washed with the same amount of PBS, and 150 μL of LF at concentrations of 0.5, 1, 2, 5 and 10 mg mL^{-1} diluted in the basic medium were added. Two wells were assigned as controls, which consisted in cells incubated only with basic medium without LF. The plate was incubated for 1 h at 37 °C. After incubation, cells were washed again with PBS, and 50 μL per well of a 4–5 log cfu mL^{-1} *C. sakazakii* suspension were added (also in control wells) to 150 μL per well of basic DMEM, and incubated for 4 h at 37 °C. Then, cells were washed and 200 μL per well of 150 μg mL^{-1} gentamicin were added, incubating them for 2 h at 37 °C to inactivate the bacteria that have remained outside the cells, which were eliminated with a final washing.

Afterwards, the plate was incubated with 200 μL per well of trypsin for 15 min at 37 °C to permeabilise and detach the cells. Finally, serial dilutions of the suspensions with peptone water were spread with Digralsky handle on TSA plates. The plates were incubated for 24 h at 37 °C and the colonies were counted.

Table 1
Primer sequences used for qPCR analysis of the expression of housekeeping genes, TLR and LF receptor (LFr) in Caco-2/TC7 cells.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Reference
GAPDH	CATGACCACAGTCCATGCCATCACT	TGAGGTCCACCACCCTGTTGCTGTA	Buey et al. (2021)
HPRT1	CTGACCTGCTGGATTACA	GCGACCTTGACCATCTTT	Buey et al. (2021)
TLR2	GAAAGCTCCCAGCAGGAACATC	GAATGAAGTCCCGCTTATGAAGACA	Buey et al. (2021)
TLR4	TTGAGCAGGTCTAGGGTGATTGAAC	ATGCGGGACACACACACTTTCAAATA	Buey et al. (2021)
TLR9	AGTCCTCGACCTGGCAGGAA	GCGTTGGCGCTAAGGTTGA	Buey et al. (2021)
LFr	AATGGACCTGTTCTTCGT	TCTGGGTAGACTGCTTTG	Zheng et al. (2012)

2.4.4. Oxidative stress assays

The oxidative stress in proteins and lipids was evaluated in Caco-2/TC7 cells cultured with *C. sakazakii* and LF. To perform these assays, the cells were cultured in 6-well plates at a density of 1×10^5 cells per well for 15 days and the same protocol described above in the inhibition of bacterial internalisation assay was followed to the step of incubation with bacteria. Two control wells were included, one without LF and bacteria, and one only with bacteria. The LF concentrations evaluated were 0.5 and 10 mg mL^{-1} assayed in duplicate in three independent experiments.

After 4 h incubation with bacteria, as described in the inhibition of bacterial internalisation assay, cells were washed twice with PBS and 200 μL per well of cold Tris-mannitol buffer were added. This buffer is composed of 200 mM Tris, 500 mM mannitol, 2% (v/v) sodium azide, 100 mM phenylmethylsulfonyl fluoride (PMSF), 1 pill of protease inhibitor and 25 mg mL^{-1} of benzamidine, pH 7.1 (all reagents from Sigma–Aldrich).

Next, the cell monolayer was scraped over with a scraper brush and the cells were taken to a homogeniser. The homogenate was kept on ice and disrupted by sonication (15 × 1 s bursts, 60 W). Then, the homogenate was centrifuged at 3000×*g* for 10 min at 4 °C and the supernatant, corresponding to the cell homogenate, was collected to analyse lipid and protein oxidation.

The protein content of cell homogenates was determined by the Bradford method and protein oxidation was analysed by the measurement of carbonyl levels (Latorre el at., 2014). For this purpose, the optimum concentration of protein was 0.5 mg mL^{-1}. The samples were analyzed in triplicate. Cell homogenates were incubated with 2,4-dinitrophenylhydrazine (DNPH), a carbonyl reagent, and protein carbonylation was measured at 375 nm. Results were calculated in nanomoles of carbonyl groups per mg of protein and expressed as the percentage respect to the control value (100%).

Lipid oxidation was assayed as described in Latorre et al. (2014). The oxidative stress in lipids was determined by measuring the concentration of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA), which reacted with N-methyl-2-phenyl-indole, resulting in a chromophore, whose absorbance was measured at 586 nm. Results were calculated in nanomoles of MDA + 4-HDA per mg of protein and expressed as the percentage respect to the control value (100%).

2.4.5. RNA extraction, reverse transcription and real-time PCR

TLR and LF receptor (also known as intelectin-1) expression was determined in Caco-2/TC7 cells cultured for 15 days in 24-well plates at a density of 3×10^4 cells per well, following the same protocol of inhibition of *C. sakazakii* internalisation to the step of gentamicin addition. Cells were incubated (24 h or 1 h) with samples of LF at 0.5 and 10 mg mL^{-1}, with and without *C. sakazakii*. Four wells were included as controls without LF, two with bacteria and two only with cells. Additionally, two wells were assigned as positive control, with 30 and 60 μg mL^{-1} of LPS from *Escherichia coli* (Sigma–Aldrich). All samples were assayed in duplicate in, at least, two independent experiments.

mRNA was extracted from cells using the RNeasy mini kit (Qiagen, Hilden, Germany), following manufacturer's recommendations. The complementary DNA (cDNA) was obtained by the action of reverse retrotranscriptase using the qScrit cDNA SuperMix kit (Quantabio, Beverly, MA, USA). Samples were taken to a thermocycler (Bio-Rad Laboratories, Madrid, Spain) and were incubated for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C, maintaining the samples at −20 °C until subsequent use. The cDNA obtained was amplified with the Real-time Polymerase Chain Reaction technique (qPCR) in qPCR Step One equipment (Applied Biosystems, Foster City, CA, USA), using SYBR Green Master Mix (Applied Biosystems) to determine the expression of TLR2, TLR4, TLR9 and LF receptor genes. GAPDH and HPRT1 were used as housekeeping genes. The specific primers are detailed in Table 1.

The results obtained for the threshold cycles (Ct) were statistically analysed by subtracting the mean of the Ct values corresponding to the housekeeping genes from the Ct for amplification of TLR genes ($\Delta Ct_{treatment} = Ct_{gene} - Ct_{housekeeping}$). The mean values of the negative controls were subtracted from the previously obtained value ($\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$). Finally, the relative gene expression was calculated and expressed as fold difference ($2^{-\Delta\Delta Ct}$).

2.5. Statistical analysis

The results are presented as the mean ± standard deviation. Statistical analysis of the results was performed using the statistical software GraphPad Prism v8.0.2 (GraphPad Software, San Diego, CA, USA). The normality of data was checked with the Shapiro–Wilk test. To compare the means of three or more unpaired groups, an analysis of variance (ANOVA) was performed and Dunnet's test was used as a multiple comparison test. Data that did not follow a normal distribution were submitted to the non-parametric Kruskal–Wallis test followed by Dunn's test as a multiple comparison test. Differences with a p-value ≤ 0.05 were considered statistically significant.

3. Results and discussion

LF is known as a multifunctional protein with several properties, including antibacterial activity among them (García-Montoya et al., 2012). It is also involved in some immunological processes, as it can inhibit inflammation or promote the innate and adaptive responses of the immune system (Actor et al., 2009). Different approaches have been carried out in this study to evaluate the bioactivity of LF against *C. sakazakii* in a model of human intestinal cells.

3.1. Antibacterial activity of lactoferrin against C. sakazakii

Based on the antibacterial activity of bovine LF, we have studied its effect against *C. sakazakii*, an emerging pathogen associated with some food products, such as powdered infant formula (Iversen & Forsythe, 2003). In particular, LF is being used as a supplement in infant milk products to mimic the composition of human milk and enhance child defences (Telang, 2018). Moreover, the presence of LF

can be useful to prevent the contamination of infant formula with some pathogens, such as *C. sakazakii* (Harouna et al., 2020).

The high affinity of LF for bacterial LPS is well known and, consequently, bovine LF can be contaminated with this endotoxin, as it is isolated from milk that contains a wide variety of bacteria (Drago-Serrano, De La Garza-Amaya, Luna, & Campos-Rodríguez, 2012). Therefore, although we were aware of the high quality of the LF used in our assays, the possible presence of LPS bound to LF was investigated. The level found was of 8×10^{-3} endotoxin units per mg protein, so the amount of LPS should be very low and, consequently, it did not affect the results of the assays.

In this study, the antibacterial activity of bovine LF against *C. sakazakii* was evaluated in different stages of bacterial growth. The results obtained in the exponential and in the stationary phases after 4 and 24 h of incubation with LF are shown in Fig. 1A and B.

As shown in Fig. 1, LF exerted a clear antibacterial activity against *C. sakazakii*, which was directly proportional to LF concentration. The bacteria were more sensitive to LF in the exponential phase and after a shorter incubation time (4 h versus 24 h). When *C. sakazakii* was in the exponential phase (Fig. 1A), treatment with LF at 1 mg mL^{-1} for 4 h caused a 1.5 log units decrease respect to the control. Furthermore, when the concentration of LF increased to 2, 5 or 10 mg mL^{-1}, the antibacterial activity was more effective, reducing the bacterial counts by 4 log units respect to the control. The antibacterial effect at longer incubation times (24 h) was only effective with statistically significant differences when the concentration of LF was, at least, of 5 mg mL^{-1}.

On the other hand, LF at 5 and 10 mg mL^{-1} reduced *C. sakazakii* growth in 3 and 2 log units, respectively, when it was in the stationary phase after 4 h of incubation (Fig. 1B). At 24 h of incubation, LF also reduced bacterial counts at the highest concentrations.

According to Embleton, Berrington, McGuire, Steward, and Cummings (2013), the antibacterial activity of LF is attributed mainly to its ability to bind free iron, preventing its use by bacteria. LF also destabilises the cell membrane of bacteria, adhering to the porins located on the surface of bacteria and causing the release of LPS, thus increasing bacterial fragility.

In our previous studies (Harouna et al., 2015, 2020), the activity of native and saturated bovine LF was evaluated against *C. sakazakii* only in the stationary phase. The results showed that native LF had a high inhibitory activity on *C. sakazakii* growth, while the iron-saturated LF did not have this effect. In addition, it was also shown that the presence of native LF in powdered infant formula was useful to minimise the growth of *C. sakazakii* when it had eventually contaminated the reconstituted formula. Our results complement these previous studies, analysing the even greater effect of native LF against *C. sakazakii* in the exponential phase of growth.

Alugupalli, Kalfas, Edwardsson, and Naidu (1995) determined the interaction between LF and *Actinobacillus actinomycetemcomitan*, a Gram-negative bacterium. They concluded that a specific interaction between LF and the bacterial outer membrane proteins occurred. They claimed that the binding of LF to bacteria was higher in the exponential phase than in the stationary phase of growth. On the other hand, in the study by Arnold, Russell, Champion, and Gauthier (1981) they also reported that the sensitivity of *Streptococcus mutans* to LF was higher in cultures at early exponential phase compared with cultures at early stationary phase, which were more resistant.

3.2. Effect of lactoferrin on Caco-2/TC7 cell viability

To carry out the different assays of antibacterial activity of LF in Caco-2/TC7 cells, it was necessary to evaluate previously the effect of LF on cell viability. This analysis allowed us to disregard the possible cytotoxic effect of LF on Caco-2/TC7 cells at the concentrations tested in the assays.

Some authors have reported that bovine or human LF promotes the proliferation of some cells, as a fibroblastic cell line (Azuma, Mori, Kaminogawa, & Yamauchi, 1989), bone forming cells, osteoblasts and cartilage cells (Cornish, 2004). In the study of Azuma et al. (1989), this stimulating effect of LF seems to be due to the iron carried by the molecule. However, in the study of Cornish (2004), the degree of iron saturation did not appear to influence the osteogenic behaviour. Furthermore, Hirotani et al. (2008) reported that human LF was able to reduce epithelial cell damage and tight junction opening in Caco-2 cells caused by bacterial LPS.

In addition, it has been reported that human recombinant LF is internalised by Caco-2 cells from the apical side, and subsequently localises in the nucleus (Ashida, Sasaki, Suzuki, & Lönnerdal, 2004). This internalisation from the apical side of Caco-2 suggests that it may be involved in iron uptake from the luminal fluid. Furthermore, the nuclear localisation of LF may indicate another function in the cells, such as modulation of intestinal function through gene regulation, as LF has been reported to bind to a specific DNA sequence (Ashida et al., 2004).

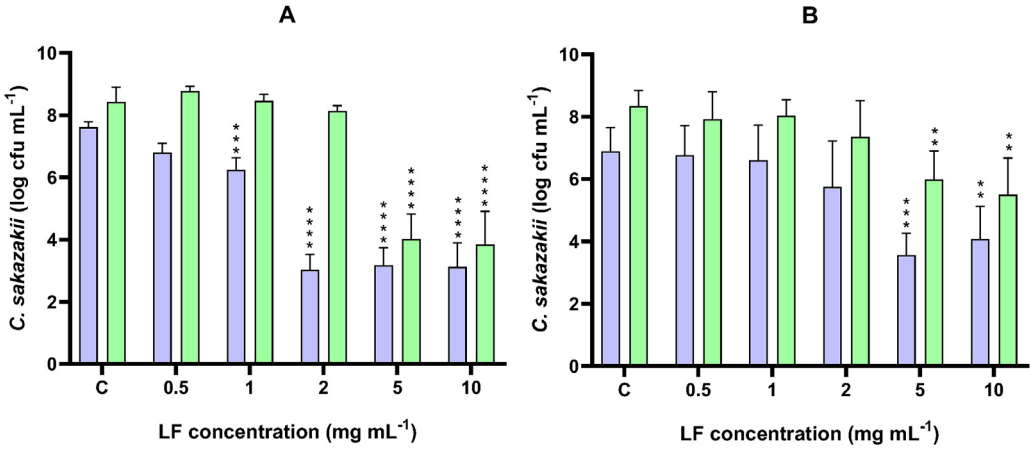

Fig. 1. Antibacterial activity of bovine LF against *C. sakazakii* in two different growth stages after incubation for 4 h (■) and 24 h (■): (A) exponential phase after 8 h growth and (B) stationary phase after 18–20 h growth. C: control without LF. The values represent the mean ± standard deviation of two replications in three independent experiments (n = 6). $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$, respect to the control.

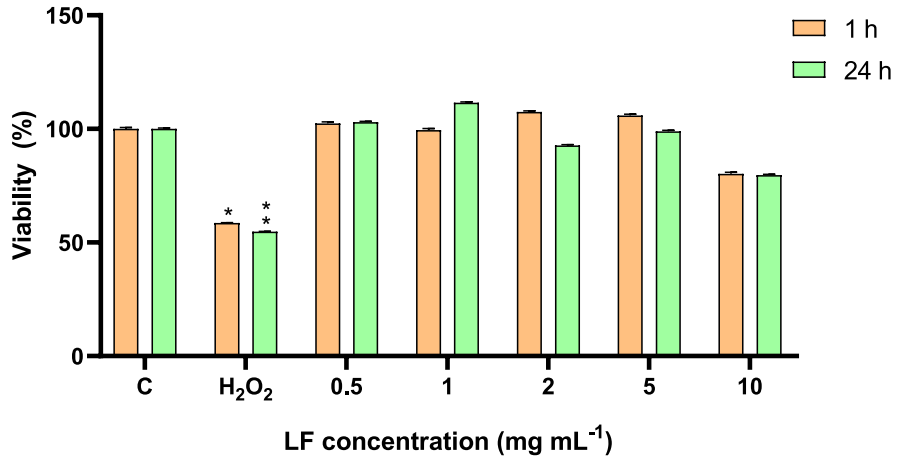

Fig. 2. Viability of Caco-2/TC7 cells after 1 h or 24 h of incubation with bovine LF. C: Control of cells without treatment. H_2O_2: positive control of cytotoxicity consisting of cells treated with H_2O_2. The viability is expressed in percentage respect to the control that consisted of cells grown in complete medium. The values represent the mean ± standard deviation of two replications in three independent experiments (n = 6). *$p < 0.05$, **$p < 0.01$ compared with the control.

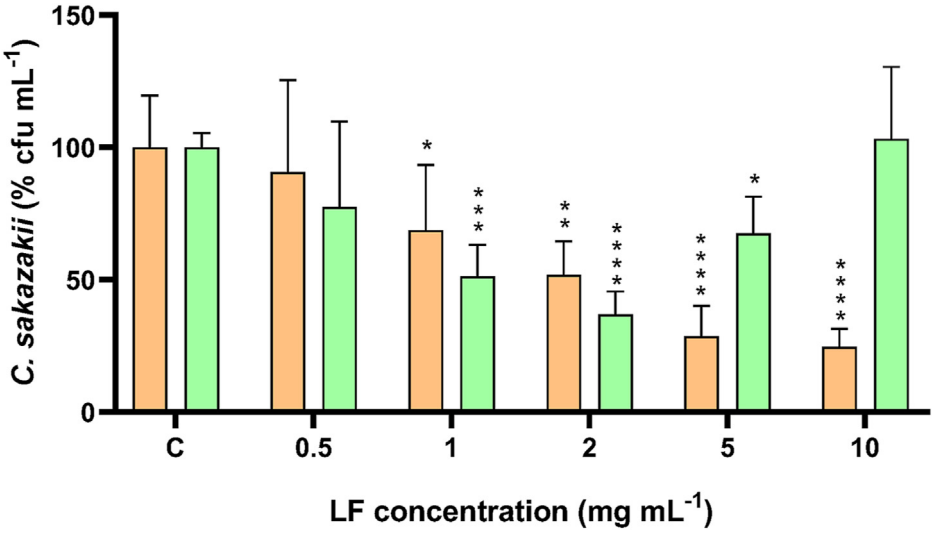

Fig. 3. Inhibition of *C. sakazakii* internalization into Caco-2/TC7 cells caused by bovine LF at different concentrations and times of incubation (■, 1 h; ■, 24 h). C: control of cells without treatment. The growth is expressed in percentage of UFC mL^{-1} respect to the control. The values represent the mean ± standard deviation of two replications in three independent experiments (n = 6). *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001 compared with the control.

With our results, we can conclude that LF does not affect the viability of Caco-2/TC7 when incubated for a maximum time of 24 h (Fig. 2), although a certain decrease in cell viability was observed at LF concentration of 10 mg mL^{-1}. In any case, this decrease did not present statistically significant differences respect to the control. These results agree with those of the study by Atef Yekta et al. (2010), which also confirmed that none of the bovine LF concentrations they assayed, up to 10 mg mL^{-1}, was cytotoxic to Caco-2 cells.

3.3. Effect of lactoferrin on inhibition of *C. sakazakii* internalisation by Caco-2/TC7 cells

The colonisation process of the intestine by a pathogen requires its adhesion to the host cells. After adhesion, the pathogen crosses the intestinal barrier and enters the bloodstream, causing the development of the disease (Ribet & Cossart, 2015). In the case of *C. sakazakii*, it has been reported that the outer membrane protein A (OmpA) plays an important role in the invasion of intestinal cells by this bacterium. It seems that *C. sakazakii* interacts with the target cell through fibronectin-mediated binding. This could be a nonspecific first step, facilitating further specific interactions between bacterial ligands and host receptors (Mohan Nair & Venkitanarayanan, 2007).

In this study, we evaluated the capacity of bovine LF to inhibit *C. sakazakii* internalisation into Caco-2/TC7 cells. This cell line derives from human colon adenocarcinoma cells and, after differentiation, expresses morphological and functional characteristics of enterocytes (Sánchez, Ismail, Liew, & Brock, 1996). For this purpose, we evaluated two different incubation times of the cells with LF, 1 and 24 h, before the addition of bacterial suspension, to test if the cells had different responses depending on the time exposed to LF.

As shown in Fig. 3, LF had an inhibitory effect on the internalisation of bacteria into Caco-2/TC7 cells. This effect was directly

proportional to LF concentration when the previous incubation time of cells with the protein was 1 h. However, when the time of cell exposure to LF was increased up to 24 h, the protective effect disappeared at high concentrations of LF. A possible explanation for this effect would be that a long exposure to LF would increase the expression of LF receptors in the cells and, consequently, the binding of LF to them, thus reducing its activity against bacteria. To verify this hypothesis, the expression of LF receptor was also determined. The results indicated that only in the cells incubated with LPS, which was used as control, there was an increase in LF receptors although it was not significant; whereas the cells incubated with LF (0.5 and 10 mg mL^{-1}), with or without *C. sakazakii*, did not over-express LF receptors (results not shown). It has been reported that Caco-2 cells express a maximum level of LF receptors about 16 days of culture (Lönnerdal, Jiang, & Du, 2011). Therefore, it is possible that at the differentiation stage of our cells the level of LF receptors cannot be further increased by LF or *C. sakazakii*.

In relation to our results, Valenti et al. (1999) demonstrated that LF has the capacity to reduce significantly the invasion of intestinal Caco-2 cells by *Listeria monocytogenes*. Furthermore, both human and bovine LF have been shown to inhibit the adherence of *E. coli* to Caco-2 cells and the inhibition is dose dependent (Atef Yekta et al., 2010).

There have also been some previous studies reporting the effect of some compounds interfering in the internalisation of *C. sakazakii* into Caco-2 cells. Thus, Quintero et al. (2011) studied the inhibition of *C. sakazakii* adhesion to Caco-2 cells mediated by galactooligosaccharides of commercial origin. They observed that galactooligosaccharides caused a 31% reduction in *C. sakazakii* adhesion, although the concentration used was very high (16 mg mL^{-1}), more than that usually added to supplement infant formula.

In a previous study, we observed that different dairy by-products, such as commercial skim milk, butterserum and buttermilk, and also raw buttermilk, inhibited the adhesion of *C. sakazakii* to Caco-2/TC7 cells at a level no higher than 22% (Ripollés et al., 2017). It was found that the specific activity of raw buttermilk (percentage of adhesion inhibition per mg of protein) was lower than that of commercial products. It is possible that the higher effect of commercial products was due to the heat treatment applied to them, as it can denature milk proteins, exposing their hydrophobic groups, which would interact with the surface molecules of bacteria, avoiding their binding to cells (Ripollés et al., 2017).

The inhibition of microorganism adhesion to host cells by LF has been previously studied. LF has been shown to have an inhibitory effect on the internalisation of viruses, such as human papillomavirus (HPV). This activity was more potent for bovine LF than for the human counterpart (Drobni, Näslund, & Evander, 2004). The same authors suggested that LF had two mechanisms to prevent the adsorption of different viruses to target cells. It could bind directly to the virus (like for rotavirus, HIV or poliovirus) or it could link to heparin sulphate proteoglycans on the cell surface, interfering in both cases with the binding of the virus to host cells.

Furthermore, both apo-LF and holo-LF have been shown to have a significant capacity of inhibiting the adhesion and internalisation of *E. coli* to human epithelial cells (Longhi et al., 1993). These authors have shown that in their experimental conditions, LF bound both to the cell membrane and the bacterial outer membrane. Another study by Kawasaki et al. (2000), also confirmed that bovine LF had an adhesion-inhibiting activity against different *E. coli* strains. This capacity was tested in vitro on human epithelial cells (JTC-17) and in vivo on intestinal mucosa of mice. These authors confirmed that the inhibitory effect of LF was concentration dependent, which agrees with our results.

Finally, Quintero-Villegas, Wittke, and Hutkins (2014) performed assays evaluating the adherence of *C. sakazakii* to Hep-2 cell line. They observed that the adherence of this bacterium to those cells decreased in presence of LF. This effect was observed in a percentage of 80–99% of efficiency at a minimum LF concentration of 10 mg mL^{-1} incubated for 30 min, without additional significant effect with higher concentrations, similar to the results we have found in Caco-2/TC7 cells (Fig. 3).

3.4. Effect of lactoferrin on oxidative stress induced by *C. sakazakii* in Caco-2/TC7 cells

Some studies have shown that the presence of pathogens, such as *Helicobacter pylori*, in the intestine can produce ROS enzymes and subsequently, oxidative stress (Ivanov et al., 2017; Naito & Yoshikawa, 2002). Furthermore, LPS, present in the outer membrane of Gram-negative bacteria, has been reported to increase the protein and lipid oxidation on cells (Latorre et al., 2014). For this reason, it is of great interest to find natural substances that can avoid or decrease cellular oxidative stress. In this study, the effect of LF was analysed on the oxidative stress possibly induced in Caco-2/TC7 cells by a stressor as it can be a pathogen like *C. sakazakii*.

In our previous study of Buey et al. (2021) the effect of LF, incubated for 24 h, was evaluated on the basal oxidative stress of Caco-2/TC7 cells. When the cells were incubated with LF, it did not seem to modify by itself the lipid or protein oxidative status of

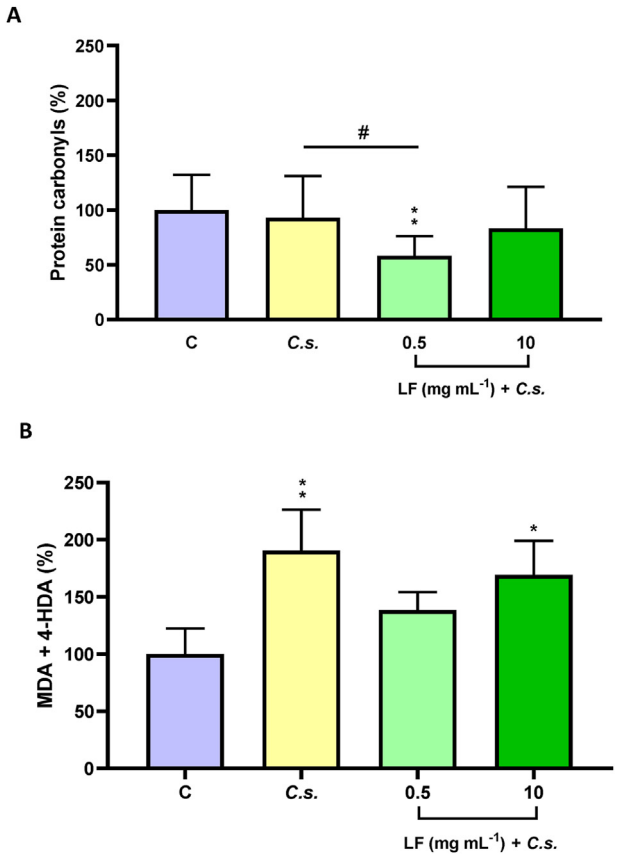

Fig. 4. Effect of bovine LF on oxidative stress caused by *C. sakazakii* in Caco-2/TC7 cells. (A) Protein oxidation, analysed by the measurement of carbonyl level. (B) Oxidative stress in lipids, determined by the concentration of MDA+4-HDA. C: control of cells without LF or *C. sakazakii*; C.s: cells treated with *C. sakazakii* without LF. The values represent the mean ± standard deviation of two replications in three independent experiments (n = 6). *$p < 0.05$, **$p < 0.01$ compared with the control. #$p < 0.05$ compared with *C.s.*

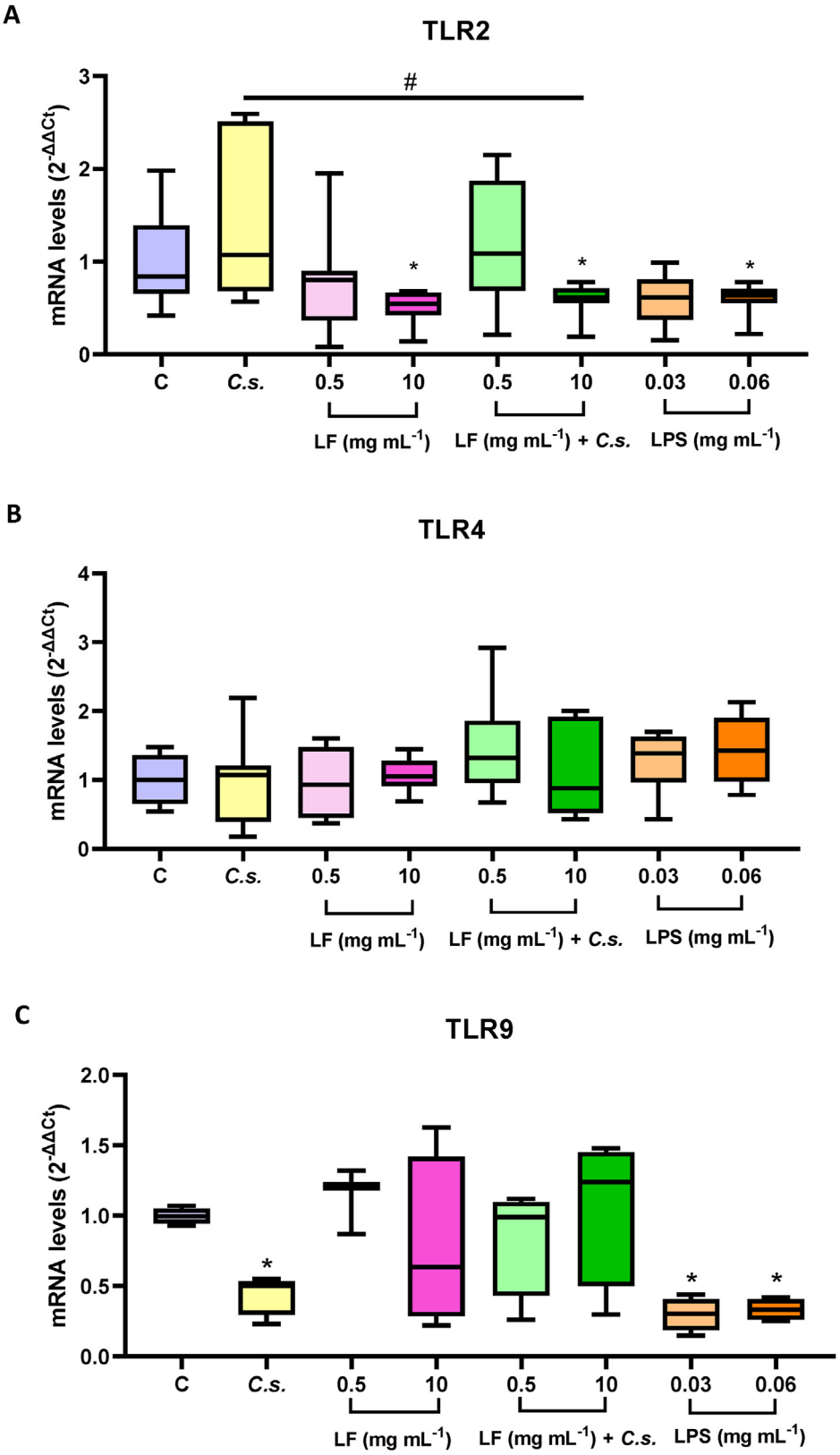

Fig. 5. Effect of bovine LF at two concentrations (0.5 and 10 mg mL^{-1}) on the expression of (A) TLR2, (B) TLR4 and (C) TLR9 in Caco-2/TC7 cells. Incubation of LF with cells for 1 h. C: control of cells without LF and without *C. sakazakii; C.s*: cells with *C. sakazakii* without LF; LPS: cells with LPS without LF. The values represent the mean ± standard deviation of two replications in, at least, two experiments (n ≥ 4). *$p < 0.05$ compared with the control. #$p < 0.05$ compared with *C.s.*

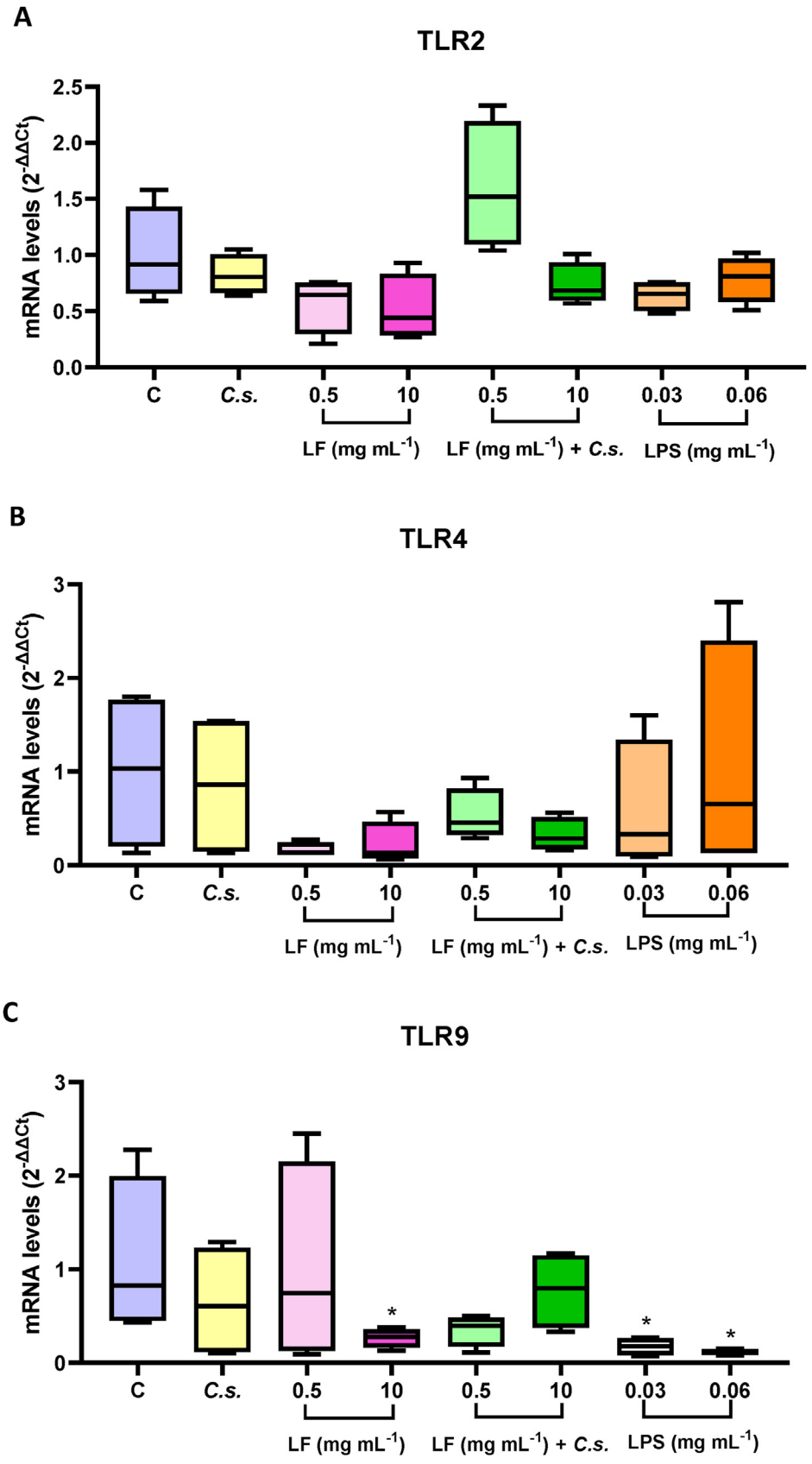

Fig. 6. Effect of bovine LF at two concentrations (0.5 and 10 mg mL^{-1}) on the expression of (A) TLR2, (B) TLR4 and (C) TLR9 in Caco-2/TC7 cells. Incubation of LF with cells for 24 h. C: control of cells without LF and without *C. sakazakii; C.s*: cells with *C. sakazakii* without LF; LPS: cells with LPS without LF. The values represent the mean ± standard deviation of two replications in two experiments (n = 4). *$p < 0.05$ compared with the control.

Caco-2/TC7 cells. Moreover, according to Zhao et al. (2019), LF decreases paracellular permeability and increases the activity of alkaline phosphatase and transepithelial electrical resistance, strengthening the barrier function of intestinal epithelium.

The incubation of Caco-2/TC7 cells with *C. sakazakii* caused a clear and significant increase in the oxidative stress in the lipid fraction (Fig. 4B). The treatment of cells with LF allowed a decrease of this oxidative stress caused by the bacteria. However, this pathogen did not seem to alter the oxidative stress in proteins in the same way (Fig. 4A), at least for the incubation time of 4 h. In any case, treatment of the infected cells with LF at low concentrations (0.5 mg mL^{-1}) reduced significantly the presence of carbonyls (Fig. 4A). These results agree with those obtained in the study by Buey et al. (2021), in which LF showed a positive effect in reducing oxidative stress caused to the cells by LPS. However, in our study, the protective effect of LF against *C. sakazakii* was greater at lower concentration (0.5 mg mL^{-1}), while in the study by Buey et al. (2021), the concentration of 10 mg mL^{-1} was the one with greater effect reversing oxidation caused by LPS.

In the study by Liu et al. (2019), human recombinant LF showed an effect of down-regulation of some inflammatory markers and upregulation of cell proliferation when Caco-2 cells were treated with hydrogen peroxide to mimic epithelial damage during intestinal injury.

3.5. Effect of lactoferrin on TLR expression

Some studies show that oxidative stress and liberation of ROS are related with the activation of TLR pathways. In the study by Yoshino and Kashiwakura (2017), ROS induced by radiation promoted the expression of TLR2 and TLR4, while Latorre et al. (2014) showed that the activation of these receptors improved the oxidative status of intestinal epithelial cells.

Milk proteins and LF in particular, have been reported to be involved in processes of inhibition and promotion of cellular immune responses (Legrand, Elass, Carpentier, & Mazurier, 2005). Puddu et al. (2011) demonstrated that the induction of IL-6 mediated by bovine LF was affected when TLR2 and TLR4 of human monocytes were blocked with antibodies, which indicates a critical role of LF in modulating host immune function.

In this study, the effect of LF on Caco-2/TC7 cells after incubation with *C. sakazakii* was analysed by determining the expression of TLR2, TLR4 and TLR9, using LPS as an internal and comparative control. We found that LF decreased the expression of TLR2 at short and long incubation times, being statistically significant respect to the control when LF was added at a concentration of 10 mg mL^{-1} after 1 h of incubation (Figs. 5A and 6A). This decrease can be explained by the fact that there was no active inflammatory process, since TLR2 is believed to have a role in ameliorating intestinal injury induced by chronic inflammatory processes (Burgueño & Abreu, 2020). However, in the presence of bacteria, differences were observed between 1 h and 24 h exposure to LF, with significant differences observed only at short-term. This effect could be due to the activation of compensation mechanisms in the 24 h incubation assay. The infection of the cells with *C. sakazakii* caused an increase in TLR2 expression, which was reversed by LF, causing a significant decrease at 10 mg mL^{-1} (Fig. 5A).

With respect to TLR4 expression, the results obtained were different depending on the exposure times with LF, showing a marked decrease in the expression of TLR4 when the cells were incubated with LF, with or without bacteria, for 24 h (Fig. 6B). These results agree with those obtained by Buey et al. (2021), in which LF significantly decreased the expression of this receptor after 24 h of exposure. However, this decrease was not observed when the cells were incubated with LF only for 1 h (Fig. 5B). The results observed with LPS, used as a comparative control, showed a marked activation of TLR4 expression after 24 h of incubation, showing that a prolonged time of exposure to bacteria or to LPS endotoxin enhanced the cell response. In fact, TLR4 has been reported to recognise cellular components of bacteria, such as LPS, having a protective effect in gut injuries (Burgueño & Abreu, 2020).

In other cell types, the effect of LF reported on TLR4 expression is different from that found in our study. Thus, Figueroa-Lozano, Valk-Weeber, Leeuwen, Dijkhuizen, and Vos (2018) showed that LF induced strong activation of TLR4, being the protein core, without attached glycans, the part of the molecule responsible for the stimulation of this receptor. In addition, Na et al. (2004) suggested that LPS was required for TLR4 signalling in macrophages and it was part of the immunomodulatory function and antimicrobial activity of LF. According to this, in the present study we found that LPS tended to increase the expression of TLR4 (Fig. 6B). In any case, the mechanisms of LF influence on TLR4 expression can be very different depending on the cell type.

As far as TLR9 is concerned, the presence of *C. sakazakii* or LPS in the cells produced a significant decrease in the expression of this receptor (Fig. 5C). However, when treating the cells with LF for 1 h prior the addition of bacteria, the expression of TLR9 began to increase, reversing their effect (Fig. 5C). On the other hand, LF diminished significantly the expression of TLR9 in the absence of bacteria when Caco-2/TC7 cells were incubated with the highest protein concentration for 24 h (Fig. 6C). This receptor plays an important role in one of the main pathways responsible for anti-inflammatory effects, being able to neutralise the inflammatory signals induced by other TLRs (Vijay, 2018). In the study by Buey et al. (2021), LF induced a slight increase in the expression of TLR9, which might help in the anti-inflammatory effects.

4. Conclusions

Milk is a source of bioactive proteins, LF among them, which is considered a multifunctional protein, with very interesting properties to be a potential ingredient for functional foods and in special for protecting the newborn.

The results derived from this study indicate that LF is a dairy protein with a potential protective effect against *C. sakazakii.* This pathogen has been mainly associated with the consumption of infant formula and dairy powders. It interacts with intestinal epithelial cells, causing intestinal inflammation and villus alterations. We have found that LF presents antibacterial activity in both exponential and stationary phases of bacterial growth.

Furthermore, LF inhibits the internalisation of the pathogen into Caco-2/TC7 cells, a model of human intestinal cells. This study has also shown the effect of LF reversing the oxidative stress caused in the lipid fraction of cells infected with *C. sakazakii*. Additionally, LF modifies the expression of TLR and in particular decreases the overexpression of TLR2 caused by the pathogen when the cells are incubated for 1 h with LF previously to the infection with *C. sakazakii*. This study suggests that LF has a great value and can be a potential ingredient for functional foods. However, further experiments should be performed to improve our knowledge about the influence of dairy proteins, and LF in particular, on the innate immune system.

Credit author statement

Inés Abad: Writing – original draft preparation, Methodology, Investigation, Data curation; Andrea Sangüesa: Methodology, Investigation, Software, Data curation; María Ubieto: Methodology,

Investigation, Software, Data curation; Juan J. Carramiñana: Conceptualization, Methodology; María D. Pérez: Writing – review & editing Reviewing and Editing; Berta Buey: Methodology; José E. Mesonero: Methodology, Writing – review & editing Reviewing and Editing; Laura Grasa: Methodology, Writing – review & editing Reviewing and Editing; Lourdes Sánchez: Conceptualization, Supervision, Writing – review & editing Reviewing and Editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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