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Evaluation of in vitro antirotaviral activity of lactoferrin from different species using a human intestinal model

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ABSTRACT

Rotaviruses are the leading agents of severe diarrheal disease in infants and young children and complementary strategies to vaccination are needed. Therefore, we evaluated the activity of lactoferrin (LF) from different species against rotavirus in differentiated Caco-2/TC7 cells. The mechanism of rotavirus inhibition and the influence of iron saturation and sialic acid content were also investigated. Furthermore, the effect of bovine LF on Toll-like receptor expression (TLRs) during rotavirus infection was studied. LF presented high inhibitory activity against rotavirus above 8 mg mL^{-1}, the bovine protein being the most potent. The antirotaviral mechanism of LF was mainly related to the virus neutralisation at an early step of infection. Interestingly, a direct relationship between rotavirus neutralisation activity and content of sialic acid in LFs was observed. Finally, the neutralisation of rotavirus by LF leaded to a decreased expression of TLRs in Caco-2/TC7 cells, with a more evident effect on TLR7.

1. Introduction

Rotaviruses (RV) belong to the family Reoviridae and are non-enveloped double-stranded RNA viruses that consist of eleven distinct genome segments (Salgado, Upadhyayula, & Harrison, 2017). These pathogens primarily infect the mature enterocytes in the mid and upper part of the villi of the small intestine epithelium (Lundgren & Svensson, 2001). RV are reported as the global leading cause of acute and severe diarrhoeal diseases in both infants and young children (Tohmé & Delgui, 2021).

Rotarix ™ and RotaTeq™ are presently the two most widespread vaccines, licensed since 2006 in more than 100 high- and middle-income countries worldwide, as part of Expanded Program of Immunisation schemes and Rotavac™, RotaSIIL™ and Rotavin™ are also licensed in several countries (Hallowell, Tate, & Parashar, 2020). Even though benefits from the vaccine implementation are unquestionable, RV vaccination does not reach to all children in countries of low socio-economic conditions (Caddy, Papa, Borodavka, & Desselberger, 2021). Reasons for this, include financial and logistic problems, low availability, and impaired performance of oral vaccines (Parker et al., 2018). A systematic review of the past 10 years' literature about safety of RV vaccines in preterm infants, found that mild to moderate adverse effects have occurred in 10–60% of vaccinated infants (Sicard, Bryant, Muller, & Quach, 2020). Therefore, the importance of finding alternative strategies to confront rotaviral infection is critical and continuously underlined by the scientific community (Amimo et al., 2021).

Milk is one of the most important biofluids in nature because of its bioactive properties, being the main source of nutrition for the newborn (Muthukumaran, Mudgil, Baba, Ayoub, & Maqsood, 2023). Milk has adapted over the years to provide optimal nourishment for infants by supporting their growth and protecting them from diseases (Andreas, Kampmann, & Le-Doare, 2015). Transfer of bioactive molecules from the mother via her milk is essential for the infant, as these bioactives contribute to the maturation of the immune system, suppress inflammation, and support colonisation of healthy microbes in the gut (Ballard & Morrow, 2013). Milk components from various species have been extensively investigated through the years and numerous studies have confirmed the antimicrobial activity of some of them (Morniroli et al., 2021).

Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein found predominantly in milk, whose role in diverse biological functions is now widely accepted by the scientific community (Darmawan, Karagiannis, Hughes, Small, & Hung, 2022). During the past

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decades, it has been repeatedly shown that LF is actively involved in various physiological and protective actions, among which some of the most studied are the antioxidant, anti-tumour, anti-inflammatory and antimicrobial activities (Niaz et al., 2019; Sánchez, Calvo, & Brock, 1992). Furthermore, LF possesses significant antiviral activity against a broad spectrum of DNA and RNA viruses (i.e., herpes simplex virus, human papillomavirus, human immunodeficiency virus and rotavirus) (Kell, Heyden, & Pretorius, 2020). Studies in children under 5 years of age with rotaviral gastroenteritis reported that oral administration of LF resulted in a decrease in the severity and duration of the diarrhoeal symptoms (Zavaleta et al., 2007) and, overall, ameliorated the acuteness of the disease (Egashira, Takayanagi, Moriuchi, & Moriuchi, 2007). It has been proposed that LF exerts its antiviral activity through several types of mechanisms, as it obstructs different phases of the viral life cycle by directly inactivating virions before infection or by inhibiting its replication inside the host cell (Seganti et al., 2004). Additionally, LF has the ability to stimulate the host immune responses, leading to the production of pro-inflammatory cytokines and the maturation of T and B cells, causing an overall protective effect (Kowalczyk et al., 2022).

It is well known that characteristics such as composition, structure, and physicochemical properties of milk vary between different mammalian species (Roy, Ye, Moughan, & Singh, 2020). This is mainly due to the differences in the nutritional requirements of the newborn, depending on the degree of maturity at birth, growth rate, energy needs and environmental conditions, among other aspects (Skibiel, Downing, Orr, & Hood, 2013). As a consequence, the LF profile in milk is also species-specific, with variations observed in concentration, structure and post-translational modifications, among others (Albar, Almehdar, Uversky, & Redwan, 2014). One example of LF heterogenicity among species is its glycosylation status (Zlatina & Galuska, 2021). Bovine, caprine and ovine LF sequence reveals five possible *N*-glycosylation sites, human LF contains three potential sites, camel LF contains four, whereas murine LF has only one potential *N*-glycosylation site (Baker & Baker, 2009; Kappeler, Farah, & Puhan, 1999). Interestingly, the formation of disulphide bonds in camel LF are identical with those found in human and mare LF, but the locations of predicted glycosylation sites are entirely different in camel LF (Khan et al., 2001). Moreover, LF from caprine and bovine milk is characterised by high manose glycans, which seem to be absent in LF from human and mouse milk (Spik, Coddeville, & Montreuil, 1988). Differences have also been observed among species, within structures containing fucose and sialic acid (SA), with human LF showing a higher percentage of fucosylation and lower percentage of sialylation compared with caprine and bovine LF (Karav, German, Rouquié, Le Parc, & Barile, 2017). The contribution of *N*-glycans to the biological function of LF is not yet fully elucidated; however, there are reports that associate the structure of the glycans with cell adhesion and activation of specific receptors that finally lead to host protection against microbial and viral threats (Barboza et al., 2012; Nwosu et al., 2012).

Toll-like receptors are an important group of molecules that constitute the first line of defence system against microbes in the intestine (Sadik, Shaker, Ghanem, Hassan, & Abdel-Hamid, 2015). They belong to the family of pattern recognition receptors (PRRs), and they are crucial in host protection against viral infections as they recognise specific pathogen associated molecular patterns (PAMPs) and generate innate immune response (Vidya et al., 2018). Detection of the viral presence activates several signalling cascades in the host immune cells that result in the expression of proinflammatory cytokines, type I interferons and interferon stimulated genes to orchestrate the antiviral response and promote inflammation (Li & Wu, 2021). However, overactivation of TLRs or their downstream components disrupts the immune homeostasis by sustained production of proinflammatory agents and causes irreversible changes in organ structure and function (El-Zayat, Sibaii, & Mannaa, 2019). It has been found that elevated levels of inflammatory cytokines are in direct correlation with the severity and frequency of diarrheal episodes during the acute phase of RV infection in children (Jiang et al., 2003; Wang et al., 2007). In this view, Bellés et al. (2022) have investigated the effect of LF on the immune system through TLR interference using a murine model of induced intestinal dysbiosis. It was found that bovine LF was able to restore the normal expression levels of TLR8, TLR9 and TLR5.

The aim of the present study was to evaluate the activity of LF from bovine, human, and camel origin, as well as recombinant human LF from rice, by measuring their capacity to inhibit the infection of human enterocytes by the WC3 strain of bovine RV. The antiviral effect was tested in the human colon adenocarcinoma cells Caco-2, expressing the differentiation phenotype of mature enterocytes, which represent the in vivo target of RV infection. Three types of infection assays were performed to assess the mechanism of action against RV: (i) RV neutralisation, (ii) cell receptor blocking and (iii) post-binding activity. Furthermore, we tested the influence of bovine LF iron saturation and SA content of the different LFs on their antirotaviral activity. Finally, we examined the expression of genes encoding four Toll-like receptors (TLR2, TLR3, TLR7, TLR8) associated with viral infections to get a better understanding on the inflammatory-immune response induced by RV and LF.

2. Materials and methods

2.1. Chemicals and samples

Mature human milk, less than 24 h old, was obtained from local healthy mothers and stored in milk bags at −20 °C. The Ethical Committee for Clinical Research of the Government of Aragon (CEICA) approved the study and donors were informed about the nature of the study before they gave their consent to participate. Raw camel milk freeze-dried powder was kindly donated by Camilk Dairy (Melbourne, Australia). The camel milk powder was reconstituted according to the manufacturer's instructions (25 g of powder milk in 240 mL of water at 37 °C) and mixed until complete dissolution.

Native bovine LF (bLF) was kindly provided by Tatua Nutritionals Company (Morrinsville, New Zealand) with an iron-saturation level below 10%. Recombinant human LF (rhLF) obtained from rice in the AsIs form (As Isolated from rice) having 68% iron saturation, was kindly provided by Ventria Bioscience (Sacramento, CA, USA).

SP-Sepharose was purchased from GE Healthcare (Uppsala, Sweden). Mini-Protean TGX gels of 4–20% polyacrylamide were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Coomassie Brilliant Blue R-250 was purchased from Serva Feinbiochemica (Heidelberg, Germany). The molecular mass marker used was PageRuler™ Prestained Protein Ladder, from 10 to 180 kDa, (Thermo Fisher Scientific). Sialic Acid Assay Kit was purchased from Biotech Life Sciences (Cambridge, UK). For the preparation of the different buffer solutions, the following reagents were used: KH_2PO_4 (99.0%), KCl (95.5–100.5%) from Panreac (IL, USA), Tween-20 (≥40.0%), Na_2HPO_4 (≥98%), CH_3COOH (99.7%), H_5CO_3 (98%) and $NaHCO_3$ (≥99.5%) from Merck (Darmstadt, Germany) and NaCl (99.5–100.5 %) from VWR (Radnor, PS, USA).

The human adenocarcinoma cell line Caco-2, clone TC7 (Caco-2/TC7) was kindly donated by the Department of Physiology of the Faculty of Veterinary Medicine of the University of Zaragoza, the Rhesus monkey embryonic kidney epithelial cell line MA104 (CRL-2378) was purchased from the American Type Culture Collection (ATCC). Bovine RV strain WC3 (R402-10) was obtained from ATCC (VR-2102). Minimal essential medium (MEM), Dulbecco's Modified

Eagle Medium (DMEM), heat-inactivated foetal bovine serum (FBS), L-glutamine, antibiotics (penicillin–streptomycin), non-essential amino acids (NEAA), amphotericin B and Trypsin–EDTA, were obtained from Gibco (Life Technologies Corporation, Paisley, UK). Specific antiserum against bovine RV obtained in lamb was kindly donated by Dr Snodgrass from Moredun Research Institute (Penicuik, UK). Donkey anti-sheep immunoglobulin antiserum conjugated with fluorescein isothiocyanate (FITC), gelatin from porcine skin and trypsin from porcine pancreas used for the RV activation, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Lactoferrin isolation

Native human LF (hLF) and camel LF (cLF) were purified using cation exchange chromatography on SP-Sepharose following the procedure by Conesa et al. (2008). In brief, 100 mL of milk were skimmed by centrifugation at 2500 × *g* for 30 min at 4 °C, diluted 1:1 with a buffer containing 0.04 M NaH_2PO_4, 0.8 M NaCl, 0.04% (v/v) Tween 20, pH 7.4, and incubated batchwise with SP-Sepharose at 4 °C overnight under gentle continuous agitation (proportion gel: sample = 2:1). Afterwards, the SP-Sepharose was packed into a column (5 × 30 cm) and washed with a buffer consisting of 0.02 M NaH_2PO_4, 0.4 M NaCl, 0.02% (v/v) Tween 20, pH 7.4, to wash out the unbound proteins. LF from different origins was eluted with a buffer containing 0.02 M NaH_2PO_4, 1 M NaCl, pH 7.4. The applied flow was 3 mL min^{-1} and the fractions collected were of 3 mL. The absorbance of the fractions was measured at 280 nm, and the fractions containing LF, identified by SDS-PAGE, were pooled. For estimating the protein concentration, an extinction coefficient of 84,540 M^{-1} cm^{-1} was used for cLF (Kappeler et al., 1999), and 120,304 M^{-1} cm^{-1} for hLF (Goldman & Green, 2015). For bLF, the extinction coefficient used was 1.27 mL cm^{-1} g^{-1} (Abad et al., 2022). Finally, LF containing solutions were dialyzed against 25 mM ammonium bicarbonate, pH 7.4, lyophilised using Heto PowerDry DW8 (Thermo Fisher Scientific, Rockford, IL, USA), and stored at −20 °C. For in vitro assays, LF was diluted in phosphate-buffered saline (PBS) consisting of 0.14 M NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 8.5, and sterilised by filtration through 0.22 μm pore size filter units.

2.3. Iron saturation

To evaluate the influence of the iron saturation on the activity of bLF against RV, the procedure described by Harouna et al. (2015) was followed. A solution of ferrinitrilotriacetate (FeNTA) was initially prepared by mixing a solution of 0.02 M $FeCl_3$ with an 80 mM sodium nitrile acetate (NTA), obtaining a final concentration of 10 mM Fe^{3+}, 40 mM NTA, pH 5. Next, 3 μL of FeNTA and 3 μL of 10 mM $NaHCO_3$ were added for each mg of bLF, and the mixture was incubated for 24 h at 4 °C. Afterwards, the protein was subjected to gel filtration using Sephadex G-25 chromatography (1 × 30 cm, free flow) to remove unbound iron. The resulting bLF fraction were considered 100% iron saturated.

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the procedure described by Laemmli (1970). Milk fractions and proteins were analysed using 4–20% polyacrylamide gels, which were afterwards Coomassie stained, according to standard procedures, as described previously by Ripollés et al. (2015).

2.5. Sialic acid determination

To evaluate the total SA content of LF from different origins, the Sialic Acid (NANA) Assay Kit from Abcam (Cambridge, UK) was used. The procedure described by the manufacturer was followed. Briefly, solutions of LF from different species were diluted (1:1) in 4 N acetic acid and were incubated at 80 °C for 3 h to release SAs bound to the protein. Then, the mixture was neutralised with 4 N NaOH. The released SAs were collected by ultra-centrifugation through a 3000 MWCO filter. At this point a standard curve was prepared according to the manufacture instructions. Then, the samples and the different standards (50 μL) were diluted 1:1 with the reaction mix (50 μL), containing assay buffer, SA converting enzyme, SA development mix and SA probe. The mixture was incubated at room temperature for 30 min and protected from light. Finally, the absorbance at 570 nm was measured in a microplate reader (Labsystems Multiscan, Helsinki, Finland).

2.6. Cell culture

The Caco-2/TC7 cells were cultured in DMEM medium supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine 2 mM, 1% (v/v) non-essential amino acid solution, 1% (v/v) antibiotic solution (100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) and 1 μg mL^{-1} amphotericin B. The MA104 cells were cultured in MEM medium supplemented with 10% (v/v) FBS, 1% (v/v) 2 mM L-glutamine, 1% (v/v) antibiotic solution (100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) and 0.25 μg mL^{-1} amphotericin B. Both cell types were maintained in 25 cm^2 culture flasks at 37 °C in a Heraeus B5060 EK/CO_2 thermostatic incubator with 5% CO_2.

2.7. Rotavirus assays

2.7.1. Rotavirus propagation

The bovine RV WC3 strain (ATCC VR-2102) was propagated on the MA104 cell line according to previously described procedures (Parrón et al., 2018a). Briefly, confluent 75 cm^2 cell flasks were infected at a multiplicity of infection (MOI) of 0.1 with an aliquot of a RV suspension. Upon incubation at 37 °C for 1–3 d, the cytopathic effect was observed and the RV was harvested by subjecting the flasks to three cycles of freezing (−80 °C) and thawing (room temperature). The resulting lysate, containing cells and virus was clarified by centrifugation at 300 × *g* for 15 min at 4 °C to remove cell debris. The supernatant, considered the infective viral suspension, was titrated according to the procedures described by Arnold, Patton, and McDonald (2009), aliquoted in cryovials and stored at −80 °C until its use in the assays.

2.7.2. Rotavirus neutralisation assay

Caco-2/TC7 cells were seeded in 96-well plates at a density of 1.4×10^4 cells cm^{-2} and grown until differentiation (15–17 d). Cells were serum-starved 2 h prior to the RV assay in DMEM supplemented with 1% (v/v) L-glutamine 2 mM, 1% (v/v) antibiotic solution (100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) and 1 μg mL^{-1} amphotericin B. An aliquot of RV suspension was thawed and activated with trypsin (20 μg mL^{-1} final concentration) for 1 h at 37 °C. Next, LF from different species (at concentrations ranging from 1 to 10 mg mL^{-1}) was incubated with the RV suspensions (diluted in MEM to achieve a final MOI of 0.02) in a 1:1 ratio (final volume 50 μL) in a 96-well conical bottom plate, for 1 h at 37 °C. DMEM medium was used as negative control and the activated RV suspension diluted 1:1 with DMEM as positive control of infection. The contents of the incubated plate were then transferred (50 μL $well^{-1}$) to the previously prepared plate with the differentiated Caco-2/TC7 cells and incubated for 1 h at 37 °C. To facilitate

the post-adsorption infection phase, 100 μL well^{-1} of DMEM medium supplemented with 6% (v/v) FBS and 2 μg mL^{-1} trypsin was added to each well, and the plate was incubated at 37 °C for 12 h. After the infection period, cell fixation was followed, and infected cells were detected by indirect immunofluorescence (section 2.7.5).

2.7.3. Cell receptor blocking assay

The interaction of LF from different species with the cellular receptors of Caco-2/TC7 was studied to evaluate the influence that may have on the antirotavirus activity. Similar to the neutralisation assay, Caco-2/TC7 cells were seeded in 96-well plates and grown until differentiation (15–17 d). The culture medium was removed 2 h before the cell receptor blocking assay, and the cells were incubated with serum-free medium for 2 h. Next, 50 μL LF was added in different concentrations (1–10 mg mL^{-1}) and the plate was incubated for 1 h at 4 °C, to prevent the samples from penetrating the cells by endocytosis. After the incubation at 4 °C, wells were washed three times with 120 μL well^{-1} of PBS and infected with the viral suspension of bovine strain WC3 at a final MOI of 0.02, incubating for 1 h at 37 °C. After washing the wells with 120 μL well^{-1} of PBS, 100 μL of DMEM medium supplemented with 6% (v/v) FBS and 2 μg mL^{-1} trypsin were added to each well, and the plate was incubated at 37 °C for 12 h, subsequently proceeding to the determination of infection by indirect immunofluorescence (section 2.7.5).

2.7.4. Post-binding assay

This assay was performed to test whether LF from different origin was capable of inhibiting RV infection after its binding to the cell. Caco-2/TC7 cells were cultured in a 96-well plate until differentiation (15–17 d). The plate with the differentiated cells was then washed and incubated for 2 h with serum-free culture medium. Next, the medium was removed, and 50 μL well^{-1} of activated RV suspension was added (final MOI = 0.02). The plate was incubated for 1 h at 4 °C, to prevent the virus from entering the cell. Subsequently, after washing the cells with 120 μL well^{-1} of PBS, 50 μL well^{-1} of LF samples were added in different concentrations (1–10 mg mL^{-1}), and the plate was incubated for 1 h at 37 °C. Finally, the plate was washed with 120 μL well^{-1} of PBS, and 100 μL well^{-1} of DMEM medium supplemented with 6% (v/v) FBS and 2 μg mL^{-1} trypsin were added, and the plate was incubated at 37 °C for 12 h, subsequently proceeding to the determination of infection by an indirect immunofluorescence assay (Section 2.7.5).

2.7.5. Rotavirus detection by indirect immunofluorescence assay

After 12 h of the infection period, the cells were washed with 200 μL well^{-1} of sterile PBS. Cell fixation was then carried out by adding 300 μL well^{-1} of a solution of acetone:methanol:formaldehyde (1:1:1) and incubating for 3 min at 4 °C. Next, the plate was washed twice with sterile PBS and incubated with 100 μL well^{-1} of bovine anti-RV antiserum obtained in lamb (diluted 1/3000 in PBS) at 37 °C for 2 h under gentle agitation. Then, the wells were washed three times with sterile PBS and incubated with 100 μL well^{-1} of FITC-conjugated donkey anti-sheep IgG antibody (diluted 1/200 in PBS with 4% (v/v) gelatin) for 1 h at 37 °C under gentle agitation. Finally, after washing three times with PBS, the fluorescent cells were counted in the Eclipse E400 fluorescence microscope with a Nikon FITC filter, and the Zen lite 2012 image processing software. The infectivity percentages were determined by enumerating the fluorescent infected cells in each well in relation to the 100% infectivity obtained with the positive control, which consisted of the virus suspension without a neutralising agent.

2.8. Determination of TLR expression: RNA extraction, reverse transcription, and real-time PCR

TLR expression was determined in Caco-2/TC7 cells cultured for 15–17 d in 24-well plates at a density of 2.3 × 10^4 cells well^{-1}, following the same protocol of RV neutralisation to the step of the 12 h infection period. The control consisted of cells incubated with DMEM medium supplemented with 1% (v/v) L-glutamine 2 mM, 1% (v/v) antibiotic solution (100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) and 1 μg mL^{-1} amphotericin B. 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 qScript™ cDNA SuperMix kit (Quantabio, Beverly, MA, USA) according to the supplier's protocol. 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 the SYBR Green Master Mix (Applied Biosystems) to determine the mRNA expression of levels of TLR2, TLR3, TLR7, and TLR9. GAPDH and HPRT1 were used as housekeeping genes. The specific primers used are listed in Table 1. Each sample was run in duplicates in two independent experiments and then the mean Ct was determined from at least two runs. The relative mRNA expression under each experimental condition was expressed as $\Delta Ct = Ct_{gene} - Ct_{calibrators}$. Then, the relative gene expression levels were calculated as $\Delta\Delta Ct = \Delta Ct_{control} - \Delta Ct_{treatment}$, and finally converted and expressed as fold difference ($2^{-\Delta\Delta Ct}$).

2.9. Statistical analysis

Statistical analysis was conducted through the GraphPad Prism v8.0.2 software (GraphPad Software, San Diego, CA, USA) and results are presented as the mean ± standard deviation. The normality of the data was tested through the Shapiro–Wilk test. A two-tailed, unpaired Student's t-test was used for the comparison of means between same neutralising concentrations of LF from different origins. For the comparison of the mRNA expression of TLRs in differentiated Caco-2/TC7 cells, parametric One-Way ANOVA or nonparametric Kruskal–Wallis tests were applied. Means of different groups were compared by Dunnett's or Dunn's multiple comparison tests in the case of parametric and nonparametric conditions, respectively. Differences with p value $\leq$0.05 were considered statistically significant.

3. Results and discussion

3.1. Lactoferrin isolation

Several protocols have been described for the isolation of LF from milk such as: gel filtration using heparin-attached Sepharose (Al-Mashikhi & Nakai, 1987), metal-chelate affinity chromatography (Lönnerdal, Carlsson, & Porath, 1977), pepsin-coated photopolymerised sol–gel columns (Kato et al., 2004) and cation exchange chromatography with SPEC 70 SLS ion-exchange resin (RP-HPLC) (Liang, Wang, Wu, & Zhu, 2011). In this study, the isolation of LF from human and camel milk was performed using cation exchange chromatography (SP-Sepharose), which is considered a robust and reliable way of isolating this protein (Hahn, Schulz, Schaupp, & Jungbauer, 1998). The isolation profiles are shown in Fig. 1, in which one clear peak can be observed in the elution step with 1 M NaCl. However, in the case of cLF the peak appeared weaker than that of hLF, indicating lower concentration (Fig. 1A). In fact, when both skim milks were subjected to SDS-PAGE the intensity of the LF band in the camel skim milk was lower in comparison with the hLF band in the human skim milk as a band of

Table 1
Primer sequences for qPCR analysis of the expression of housekeeping genes and TLR receptors 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)
TLR3	CGGGCCAGCTTTCAGGAACCTG	GGCATGAATTATATATGCTGC	Rajalakshmy, Malathi, Madhavan, Srinivasan, and Iyer (2014)
TLR7	CTCCCTGGATCTGTACACCTGTGAG	CTCCCACAGCCTTTTCCGGAGCT	Rajalakshmy et al. (2014)
TLR8	GTCCTGGGGATCAAAGAGGGAAGAG	CTCTTACAGATCCGCTGCCGTAGCC	Rajalakshmy et al. (2014)

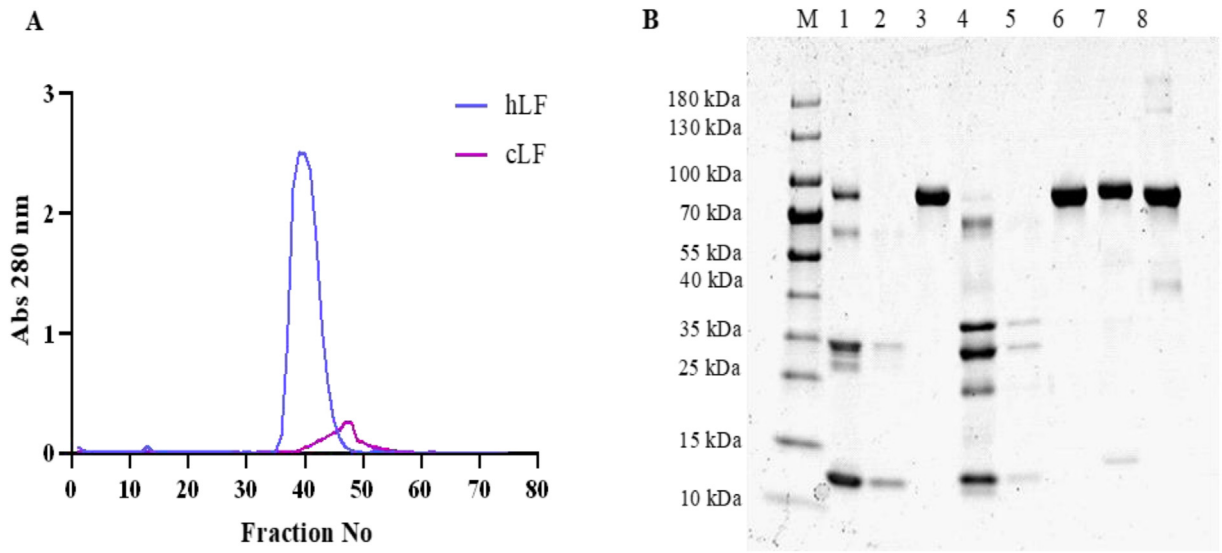

Fig. 1. Panel A, cation exchange chromatography on SP-Sepharose for lactoferrin (LF) isolation from human (blue) and camel (purple) milk. The flow rate was 3 mL min^{-1}, and the volume of the fractions was 3 mL. Panel B, Coomassie blue stained SDS-PAGE of isolated and commercial LFs, performed under reducing conditions: (M) molecular mass marker, (1) human skim milk before chromatography, (2) unbound human skim milk fraction, (3) native human LF, (4) camel skim milk before chromatography, (5) unbound camel skim milk fraction, (6) camel LF, (7) native bovine LF, (8) rice recombinant human LF.

80 kDa (Fig. 1B). All isolated and commercial LFs revealed a main band of ~80 kDa in the SDS-PAGE analysis (Fig. 1B).

3.2. Antiviral activity of LF from different species

Along the past years, LF has demonstrated antiviral activity against both DNA- and RNA viruses such as RV, respiratory syncytial virus, herpesvirus, and human immunodeficiency virus (HIV) (Berlutti et al., 2011; Chang, Ng, & Sun, 2020; Van der Strate, Beljaars, Molema, Harmsen, & Meijer, 2001; Wakabayashi, Oda, Yamauchi, & Abe, 2014). In the present study native LF isolated from human and camel milk and commercial native bovine and rice recombinant hLF, were tested for their ability to reduce RV infectivity using a model of human intestinal epithelium. The intestinal barrier was simulated using Caco-2/TC7 cells, that have the capacity to differentiate into a monolayer of polarised cells with morphological and functional characteristics of small intestine enterocytes (Ferruzza, Rossi, Scarino, & Sambuy, 2012). Concentrations of bLF up to 10 mg mL^{-1} used in this study were considered safe for the cell viability as they did not show cytotoxic effect in a study previously conducted by our research group (Abad et al., 2022). To get a better understanding of the antiviral mechanism of LF, we assessed its activity at three different stages of the virus life cycle.

The pretreatment of RV with LF (neutralisation assay) revealed that LF from human, bovine, and camel origin successfully inhibited RV infection in a dose–response manner (Fig. 2). The exception was the rice rhLF that did not present any antiviral effect at any concentration (results not shown). At the maximum concentration tested (10 mg mL^{-1}), the iron-saturated bLF marked the highest neutralisation value of activity (78.7%), having an IC_{50} of 6.18 mg mL^{-1}. The iron saturation did not seem to influence the neutralisation activity of the native bLF with an IC_{50} of 6.95 mg mL^{-1}, as the two samples demonstrated similar neutralisation profiles with no significant differences between the samples of the same concentrations. Following bLF, the camel counterpart displayed 67.9% RV neutralisation activity at a 10 mg mL^{-1} concentration. The lowest value found for neutralisation activity was that of native hLF, since it required a concentration greater than 4 mg mL^{-1} to achieve a neutralising effect, reaching a maximum of 45.7% neutralisation activity at a concentration of 10 mg mL^{-1} with an IC_{50} of 12.04 mg mL^{-1}.

The treatment of cells with LF before RV infection (cell blocking assay) resulted in minor antirotaviral activity and only in the case of native and iron-saturated bLFs (Table 2). The maximum inhibition of viral infection was 27.9% for native bLF at a concentration of 10 mg mL^{-1} and 26.9% for iron-saturated bLF at a concentration of 6 mg mL^{-1}. The rest of LFs tested were unable to reduce the RV infection (results not shown).

Finally, the application of LF after the virus adsorption to cells (post binding assay) showed a moderate effect against RV, with only native bLF and iron-saturated bLF presenting inhibition values of 50.8 and 38.8%, respectively, when added at a concentration of 10 mg mL^{-1} (Table 2). Again, none of the other LFs tested had a positive effect on RV infection (results not shown).

LF is considered an excellent candidate in the search of natural antiviral agents and one of its great advantages is the multifaced

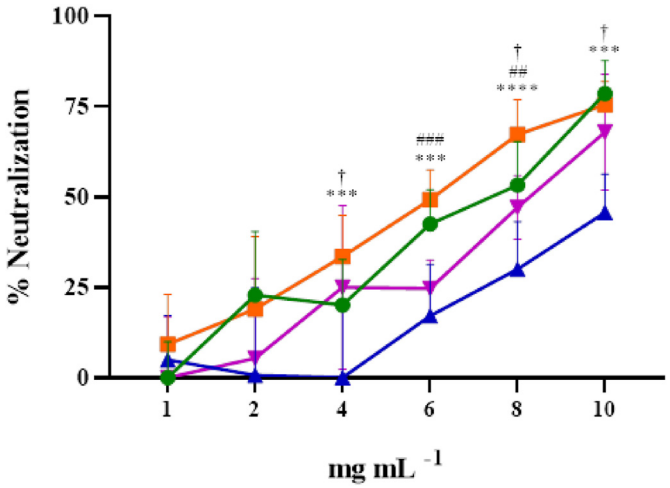

Fig. 2. Dose–response of the activity of lactoferrin (LF) from different species on neutralising bovine rotavirus WC3 strain infection of differentiated Caco-2/TC7 cells. Native bovine LF (bLF; green circle); iron-saturated bLF (orange square); native human LF (hLF; blue triangle); native camel LF (cLF; purple reversed triangle). Results are shown as mean ± standard deviation of two replicates in, at least, three independent experiments (n ≥ 6). Significant differences between the means of same concentrations are indicated with: ***$p < 0.001$ and ****$p < 0.0001$ in the case of comparison between native bLF and native hLF, ##$p < 0.01$ and ###$p < 0.001$ in the case of comparison between native bLF and native cLF and †$p < 0.05$ in the case of comparison between native hLF and native cLF.

mechanisms of action. For enteric naked viruses, such as RV, the antiviral effect of bLF is mainly exerted during the early stages of infection (Seganti et al., 2004). With regards to the neutralisation potential of LF from different mammalian origin, the results obtained here are in good accordance with the study of our research group by Parrón et al. (2018a) performed in MA104. In that study, similar intraspecies differences were reported, with bLF (both in native and iron-saturated forms) being the most effective neutralising agent among LFs from other mammals, followed by camel, and human LF. Although a similar in vitro model was applied in that study, by assessing the neutralisation activity of LF on the bovine RV strain WC3 using MA104 cells, the requirements of LF concentrations for an antiviral activity to be achieved where significantly lower compared with our study, specifically 60 times lower for bLF and 20 times lower for hLF. Assuming that LF is primarily binding to the viral capsid proteins (Parrón et al., 2018b), thus neutralising the particle, one would expect that the final infectivity observed on the cells would be similar for any tested cell line. One possible explanation for this notable difference in neutralising concentrations would be that LF, under these experimental conditions, also interacts with the cellular receptors for RV, causing an additional effect on the infection inhibition. Therefore, due to differences of cell surface receptors, the two cell lines finally respond differently to the RV/LF titre.

The study cited above by Parrón et al. (2018a) showed elevated levels of antirotaviral activity of rhLF in comparison with the native human protein, which is contradictory with the results of the present study. The polypeptide chain of rhLF and native hLF are identical and the two proteins also share similarities in their iron-binding capacity and their inhibitory activity against bacterial pathogens (Nandi et al., 2002; Suzuki et al., 2003). However, in previous studies we found some differences between rhLF and hLF in the thermodynamic parameters of their denaturation process (Conesa, Sánchez, Pérez, & Calvo, 2007) and also in their transport across Caco-2 cells (Conesa, Pocovi, Pérez, Calvo, & Sánchez, 2009). Interestingly, although rhLF and hLF both bound to Caco-2 cells and the binding constant was found similar for both proteins, the number of binding sites and the reported internalisation mechanism differed for them; this difference being attributed to their different glycosylation pattern (Nandi et al., 2002). Indeed, the post-transitional glycosylation process in plants is different from that taking place in animals. This means that in the case of rhLF expressed in rice, some terminal carbohydrate residues consist of xylose, which is not present in human systems, while SA that is a common terminal residue in native hLF is lacking (Zlatina & Galuska, 2021). Again, these differences in the sialylation maybe key to the cellular response during the RV infection in differentiated Caco-2 cells, but not in MA104 cells. In this sense, the murine RV strain EHP was described as neuraminidase (NA)-resistant when tested in the MA104 cell line (Ciarlet et al., 2002), while it behaved as NA-sensitive when tested in the human colon carcinoma cell line Caco-2 (Ludert et al., 1996), indicating that SA acid residues of LFs also may have an effect on the cell surface.

It has been reported that most of the anti-RV activity of bLF occurs during the pre-attachment and entry phase of the virus mainly through binding to viral particles and not through blocking of the cell receptors (Superti, Ammendolia, Valenti, & Seganti, 1997), but it additionally interferes with later steps during and after virus infection (Superti et al., 1997, 2001). With regards to the second less defined post adsorption mechanism of action, Seganti et al. (2004) hypothesised that the inhibition of RV infection by bLF in the post adsorption step might be related to calcium withholding, which is an essential element for the virus morphogenesis. Apart from bLF, a secondary mechanism of antirotaviral activity has not been observed in our study in the other species.

3.3. Sialic acid levels in LF from different species

Although a high degree of homology in the amino acid sequence has been reported for LF from different mammals (Chung, 1984; Le Parc et al., 2017), their glycosylation profile appears to be quite different (Nwosu et al., 2012). The distinct glycan profiles show a strong species-dependent influence on LF post-translational modifications suggesting that they could have different biological roles (Karav et al., 2017).

Table 2
Cell blocking (preventive effect) and post-binding (treatment of infected cells) activity of bovine lactoferrin (bLF) in native and iron-saturated form.[a]

Concentration (mg mL^{-1})	Cell blocking infection inhibition (%)		Post-binding infection inhibition (%)	
	Native bLF	Iron-saturated bLF	Native bLF	Iron-saturated bLF
1	0.0 ± 14.6	6.8 ± 27.3	−1.0 ± 14.6	−5.8 ± 9.7
2	3.7 ± 11.6	11.0 ± 20.6	16.1 ± 13.9	10.1 ± 30.4
4	12.9 ± 11.3	9.8 ± 26.1	28.7 ± 18.2	5.0 ± 23.2
6	12.8 ± 17.0	26.9 ± 30.3	45.9 ± 16.2	30.2 ± 8.6
8	18.1 ± 10.2	26.1 ± 9.0	35.9 ± 17.9	17.2 ± 23.4
10	27.9 ± 18.0	23.9 ± 14.9	50.8 ± 20.8	38.8 ± 16.9

[a] Results are shown as mean ± standard deviation from duplicates in at least three independent experiments.

To address the possible influence of the sialylated *N*-glycans, we estimated the sialylation degree of the LFs used in the antiviral tests, by quantitative analysis of SA released from the glycoprotein following treatment with acetic acid (Table 3). With regards to LFs isolated from milk, we observed that the bovine protein had the highest SA content, followed by camel and human LFs, with values of 3.76 (3.75 for iron-saturated), 3.07 and 2.60 μg of SA per mg of protein, respectively. The lowest content of SA was found in rhLF (0.76 μg SA per mg of LF). Prior comparative studies reported that bLF has a NeuAc content of 10 different compositions, while the human counterpart had 6 (Le Parc et al., 2017). In the same study, it was also demonstrated that 2 NeuGc glycans were additionally detected in bLF, but not in hLF. The results in our study are consistent with those of Wang et al. (2021) who found that the total SA content in native bLF was 0.462% of the protein. However, another study reported that the percentage of released SA from bLF, corresponding to Neu5Gc, was 8.5 ± 0.3% (van Leeuwen, Schoemaker, Timmer, Kamerling, & Dijkhuizen, 2012). The discrepancies among reported results might be explained by differences in the quantification protocols.

With regards to rhLF from rice, it has been reported to have the typical glycans of vegetables, such as α1−3-linked fucose and β1−2-linked xylose, and without the presence of SA residues, being different from the typical glycans of mammals, such as α2−6-linked NeuAc, β1−4-linked galactose, and α1−6-linked fucose (Fujiyama et al., 2004).

The most interesting conclusion resulting from the SA analysis is the existence of a direct relationship between the level of sialylation and the RV neutralisation potential of LF from different species. As previously described, bLF is the protein with the highest RV neutralising activity followed by cLF and hLF, in this order, with rhLF showing no neutralising activity. This order in activity corresponds to the amount of SA, from highest to lowest, present in each of the proteins.

The glycosylation has been suggested to be related with the RV infection. In that way, the entry stage of the RV infection cycle is a multistep process that initiates with the VP4 activation via trypsin cleavage to VP5* and VP8* (Crawford et al., 2001). This triggers its functional refolding on the virion surface from an upright to a reversed conformation resulting in the unmasking of the previously buried end domain for interaction with the host cell receptors (Herrmann et al., 2021). The VP8* domain contains the haemagglutination- or SA binding site that is necessary for the RV subgroup that requires SA for infection (Crawford et al., 2001; López & Arias, 2004). Although the WC3 strain is reported to be NA insensitive (Ciarlet et al., 2002) and belongs to the P(1) genogroup (Liu et al., 2012), it has been found that the VP8* domain of the bovine G6P(5) WC3 and its human-bovine mono-reassortant G4P(5) strain used in the RotaTeq vaccine, could both use α2,6-linked SA and αGal as additional ligands (Alfajaro et al., 2019).

3.4. Expression of the Toll-like receptors

The innate immune system is a highly complex biological system that provides immediate response mechanisms by recognising foreign molecular structures and triggering the activation of several intracellular signalling cascades (Nie, Cai, Shao, & Chen, 2018). This leads to the expression of several defence molecules that act as protection against infections, although under certain conditions they can have secondary harmful effects for the host itself (Cronkite & Strutt, 2018). Therefore, fine tuning of the TLR signaling cascades during viral infections is pivotal for the maintenance of homeostasis. In this sense, LF, apart from its well described direct antimicrobial role, seems to be involved in strategic points of the immune system to modulate host responses (Legrand, 2016). It has been reported that LF is able to control the expression of major molecular levers of the immune system, such as cytokines, either anti-inflammatory (e.g., interleukin (IL)-4, IL-10) or pro-inflammatory (e.g., tumour necrosis factor-α, IL-1, IL-6, IL-12) ILs and chemokines (e.g., IL-8) (Legrand, 2012). Since TLRs are described as one of major mediators of the production of these inflammatory agents upon RV infection (Omatola & Olaniran, 2022), we sought to investigate the effect of LF on their expression during the RV infection status.

Thus, differentiated Caco-2/TC7 cells were infected with RV previously neutralised with native bLF in two different concentrations. Apart from the control (cells incubated with serum-free medium), bLF alone and RV alone were also included, to observe their direct effect on the cells. Following incubation for 12 h, the expression levels of TLR2, 3, 7 and, 8 were measured using qPCR. The choice of the neutralisation assay and the native bLF was based on the optimum results that this combination yielded in the antiviral tests described in the previous section.

As seen in Fig. 3, stimulation of all four TLRs tested was marked upon RV infection, confirming that, a given pathogen may express several different PAMPs, and result in a combined recognition by multiple TLRs (Akira & Hemmi, 2003; Hatton & Guerra, 2022; Kopp & Medzhitov, 2003). It has been suggested that the activation of multiple TLRs by a single ligand is less common among plasma membrane expressed TLRs and mostly observed for endosomal TLRs, partly because they are all involved in sensing nucleic acids, and endosomes have a specific pH range that is also thought to contribute to their activation (Anwar, Shah, Kim, & Choi, 2019; Bryant et al., 2015). Overall, our results regarding the effect of the RV infection, are in accordance with those found in the study of Xu, Yang, Wang, and Jiang (2009) who have observed that RV induced increased levels of mRNA expression for TLR2, TLR3, TLR4, TLR7 and TLR8 in HT-29 cells in a time-dependent and MOI-independent manner. The same group had previously shown up-regulated expression of the same receptors in patients with acute RV diarrhoea and have positively associated the expression of TLR2, 3 and 8 with IFN-γ response (Xu et al., 2006). Non-structural protein 4 (NSP4), a transmembrane glycoprotein encoded by RV, has been identified as an additional PAMP (Ge et al., 2013). The authors showed that NSP4, purified from the medium of RV-infected differentiated Caco-2 cells, triggered the secretion of proinflammatory cytokines from macrophage-like THP-1 cells via TLR2 but not TLR3 or TLR4. In contrast, another study found elevated levels of TLR2, TLR3, TLR4, TLR5, TLR7 and TLR8, only when multiple infection of enteroviruses, including human RV Wa strain, took place; while expression of the same genes was undetectable during single RV infection (Wang, Moon, Wang, & Jiang, 2012). On the one hand, the activation of multiple TLRs may serve to reduce the demonstrated ability of RV to disrupt the host immune response (Zhu et al., 2017) by providing the host with several pathways for activating an antiviral response. On the other hand, it may result in an excessive immune reaction with detrimental effects for the host.

With respect to the role of bLF during the RV infection, we observed that preincubation of RV with bLF and subsequent infection of the differentiated Caco-2/TC7 cells resulted in

Table 3
Content of sialic acid in lactoferrin from different species.[a]

Sample	Sialic acid (μg mg^{-1})
hLF	2.60
cLF	3.07
bLF (native)	3.76
bLF (iron-saturated)	3.75
rhLF	0.76

[a] Abbreviations are: hLF, native human lactoferrin; cLF, camel lactoferrin; bLF, bovine lactoferrin; rhLF, recombinant human lactoferrin.

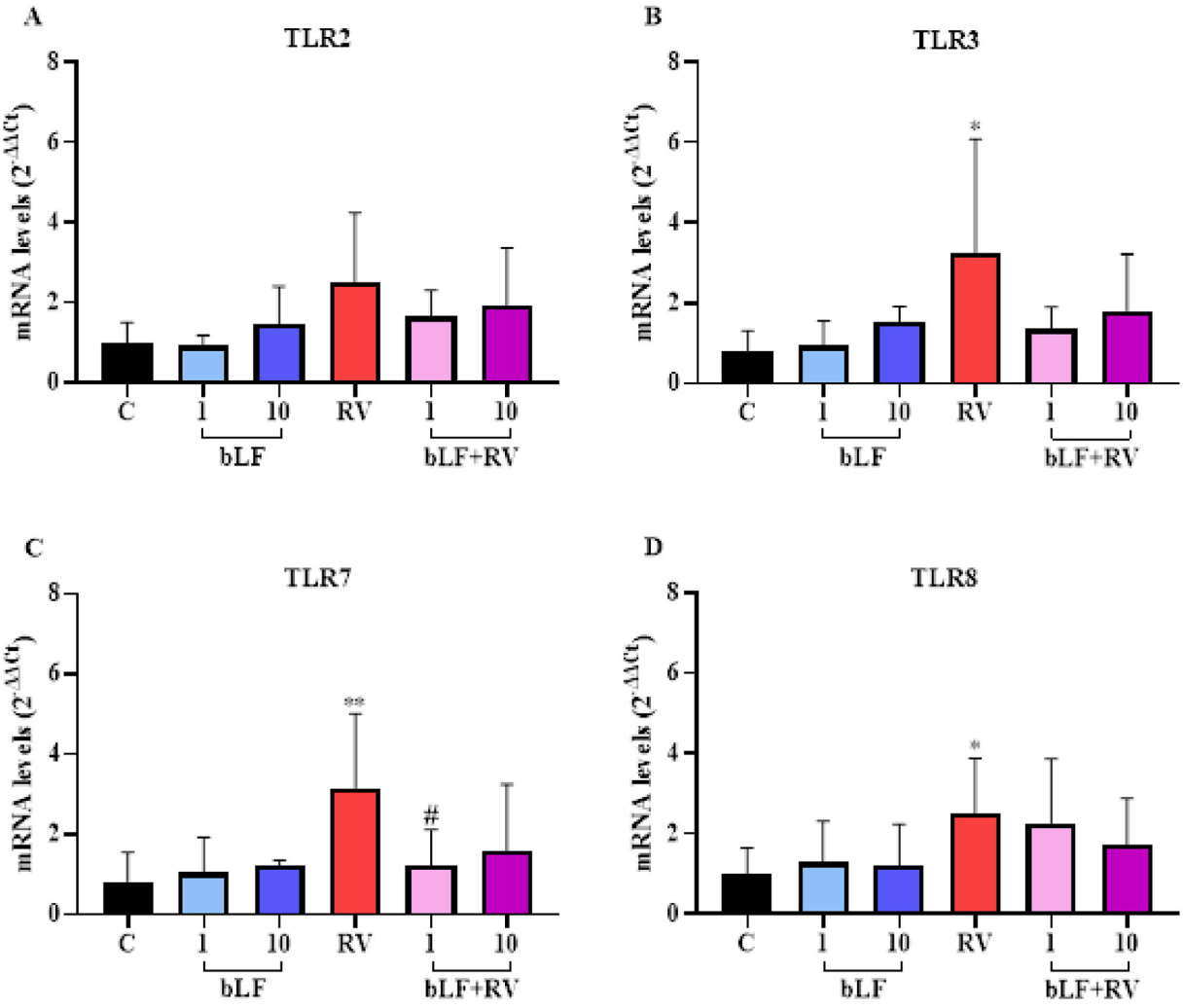

Fig. 3. Expression levels of TLR 2, 3, 7, 8 mRNA in differentiated Caco-2/TC7 cells. C: control of cells without rotavirus infection; RV: cells infected with rotavirus; bLF + RV: cells infected with RV previously neutralised by bovine LF (expressed in mg mL^{-1}) for 1 h. The values represent the mean ± standard deviation of two replicates in two independent experiments (n = 4). Significant differences of all treatments compared with the control of cells are indicated with *$p < 0.05$; **$p < 0.01$. Significant differences of cells infected with RV previously neutralised with bLF compared with cells infected with RV are indicated with #$p < 0.05$.

decreased expression of all the four TLRs tested, in comparison with the control of cells infected with RV. This effect was more evident in the case of TLR7, where the addition of bLF at 1 mg mL^{-1} to RV, significantly reduced the expression of this receptor, post infection (Fig. 3C). Interestingly, in the case of TLR2, TLR3 and, TLR8, incubation with a higher bLF concentration (10 mg mL^{-1}) lead to less reduction, showing that the modulating effect of bLF is not dose-dependent. At the same time, a slight increase of all the TLRs levels was observed when cells were incubated in the presence of bLF alone although the difference with the control was not significant. In this view, Zheng et al. (2014) showed that hLF can repress the inflammatory response induced by Epstein bar virus (EBV) in macrophages. Their results demonstrated that hLF reduced the synthesis of cytokines and antiviral mediators, such as MCP-1 and IL-8, by suppressing the NF-κB activity. Furthermore, they showed that hLF could inhibit the ability of TLR9 to recognise the viral nucleic acid by blocking the interaction between CD14 (a viral co-receptor) and TLR9.

In another study carried out in our research group, it was demonstrated that bLF can restore the normal levels of TLRs altered in a situation of intestinal dysbiosis caused by clindamycin (Abad et al., 2022). Recently, it has been shown that the glycosylation pattern of bLF determines its TLR modulating capacity (Figueroa-Lozano, Valk-Weeber, Van Leeuwen, Dijkhuizen, & De Vos, 2018). In HEK293 cells, a human embryonic kidney cell line, *N*-glycans derived from bLF strongly inhibited the activation of TLR8 and the authors attributed this fact to a direct interaction with the receptor rather than to a pH change inside the endosome (Figueroa-Lozano et al., 2020). In contrast, Ando et al. (2010) demonstrated that the carbohydrate chains of hLF were responsible for TLR4 activation. The above findings, taken together with our results on RV neutralisation, lead to the hypothesis that, in the case of the downregulation of the TLRs expression during RV infection, bLF could play a dual role: either by binding to the receptors and subsequently weakening the recognition ability of TLRs to the RV virions, or by directly binding to RV surface proteins and thus overall blocking its interaction with the host cell. These interactions seem to be glycan-mediated; however, the exact mechanism of action and the preference of bLF for the binding site, remain to be investigated.

4. Conclusions

In conclusion, this study provides evidence that LF neutralises RV infection in Caco-2/TC7 cells differentiated as human

enterocytes. This inhibition is not restricted to the neutralisation of the viral molecules, but partially extended to the suppression of the intracellular viral replication. Differences in the rotavirus neutralisation profiles of the various LFs could be attributed to the heterogenicity of the sialylation profile that characterises the protein among species. Furthermore, LF might also play a role in interfering with the host immune responses by interacting with the TLRs activation mechanism. Milk proteins and related peptides have enormous scope to be used as supplements, templates, and novel vaccine adjuvants for designing further potent antiviral drugs, but more in vivo studies are needed to evaluate their antiviral activity in depth.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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