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RESEARCH PAPER

Protective role of bovine lactoferrin in modulating the intestinal serotonergic system: Implications in intestinal inflammation

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Abstract

Bovine lactoferrin (bLf) is a multifunctional milk glycoprotein with diverse biological activities pivotal for gastrointestinal health. This study investigates the modulatory effects of bLf on the intestinal serotonergic system and its implications for intestinal inflammation and homeostasis. Intestinal serotonergic system plays a critical role in gut homeostasis, regulating various physiological processes via serotonin (5-HT) signaling. Dysregulation of this system contributes to inflammatory bowel diseases, characterized by chronic inflammation and disrupted intestinal function. We evaluated the impact of bLf on the intestinal serotonergic system *in vitro* and *in vivo*. In Caco-2/TC7 cells, bLf treatment enhanced serotonin transporter (SERT) activity and mRNA expression and modulated the mRNA levels of several serotonin receptors (5-HTRs). In mice, oral bLf administration upregulated SERT, tryptophan hydroxylase 2 (TPH2), and 5-HTRs mRNA expression in the ileum. Moreover, in a dextran sodium sulphate (DSS)-induced colitis model, bLf attenuated clinical signs of colonic inflammation, while normalizing the mRNA expression of SERT, TPH1, TPH2, and 5-HTRs in colon. These findings highlight the ability of bLf to modulate key components of the intestinal serotonergic system under both physiological and inflammatory conditions. Given the leading role of serotonin in gut-brain axis and immune regulation, bLf emerges as a promising functional food ingredient with potential for preventing or managing intestinal inflammation. Further exploration of its dietary applications may support the development of innovative nutritional strategies targeting gut-related disorders.

Keywords: Milk; Dairy; Functional foods; Serotonin, 5-HT; Colitis; Gastrointestinal health; Inflammatory bowel disease, IBD.

1. Introduction

Milk is a high-quality food renowned for its biological properties, effectively satisfying the nutritional and physiological needs of new-borns. While the milk from various species has been consumed globally for millennia, bovine milk remains the most prevalent due to its availability and production volume [1]. Research has shown that milk and dairy products can reduce the risk of developing several pathologies, including obesity [2], cardiometabolic diseases [3], type 2 diabetes [4], and certain types of cancer such as colorectal cancer [5]. The beneficial properties of milk are largely attributed to its nutritional and functional components, including proteins, lipids, carbohydrates, peptides, enzymes, vitamins, and minerals [6]. Recent evidence highlight the promise of dietary peptides and their metabolites in supporting intestinal health and broader systemic well-being, offering a foundation for the development of functional peptides aimed at enhancing human health [7].

Lactoferrin (Lf), an 80kDa glycoprotein belonging to the transferrin family, is among the key biologically active components of milk. It is naturally produced and released by mucosal epithelial cells and in the secondary granules of neutrophils in various mammalian species, including humans, cows, sheep, goats, horses, and rodents [8]. Lf is ubiquitous in biological secretions such as saliva, tears, vaginal mucus, and gastrointestinal fluids, reflecting its broad physiological significance [9,10]. In mammalian colostrum and breast milk, Lf concentrations range widely, from 4mg/ml in human milk [11] to 0.02-0.35mg/ml in bovine mature milk, depending on the lactation period [12]. Milk bovine lactoferrin (bLf), primarily derived from bovine milk, is particularly noted for its

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diverse biological properties, making it a valuable component in various commercial health products including infant milk formulas, cosmetics, and functional foods [8,13]. bLf exhibits abundant beneficial effects on gut health including immune enhancement, fortification of the intestinal barrier, and reduction of colon cancer risk [14]. bLf also acts as a prebiotic agent, modulating the gut microbiota by inhibiting pathogen growth and promoting probiotics [14]. Additionally, bLf supports iron absorption, promotes intestinal cell proliferation and maturation, and exhibits both immunomodulatory and antimicrobial activities [15].

In the context of inflammatory bowel disease (IBD), where chronic inflammation of the gastrointestinal tract leads to significant morbidity, these properties of bLf are of particular interest. IBD, encompassing Crohn's disease and ulcerative colitis, is characterized by chronic inflammation marked by periods of exacerbation and remission [16]. Its aetiology involves a complex interplay of genetic, environmental, and immunological factors [17]. Current drug therapies for IBD, including salicylates, corticosteroids, immunomodulators, and anti-inflammatories, often fall short in efficacy and carry significant side effects [18]. Therefore, novel therapeutic strategies are urgently needed.

One emerging area of research in IBD is the intestinal serotonergic system, which is essential for maintaining intestinal homeostasis. The intestinal serotonergic system involves serotonin (5-hydroxytryptamine, 5-HT), which functions as both a neurotransmitter and a neurohormone, playing a crucial role in regulating various physiological processes in the gut, including intestinal motility, secretion, absorption and sensitivity to pain [19]. Approximately 90–95% of the body's serotonin is synthesized in the enterochromaffin cells of the gastrointestinal (GI) tract, by the enzyme tryptophan hydroxylase 1 (TPH1), which converts dietary tryptophan to 5-hydroxytryptophan, the precursor of serotonin. Another isoform, tryptophan hydroxylase 2 (TPH2), is responsible for serotonin synthesis in the neurons [20]. Once synthesized, serotonin can be stored in vesicles within enterochromaffin cells or neurons and released in response to various stimuli into the gut lumen and surrounding tissue, where it can act locally or enter the circulation to exert systemic effects [21]. Serotonin exerts its effects by binding to a diverse family of 5-HT receptors (5-HTRs) expressed throughout the GI tract, mediating various responses depending on their location and type [22]. Uptake of serotonin from the extracellular space is mediated by the serotonin transporter (SERT), located in enterocytes, thus terminating serotonergic signaling. SERT is also expressed in different cells types, including enteric neurons, platelets and immune cells [23].

Dysregulation of the intestinal serotonergic system has been implicated in several gastrointestinal disorders, including IBD [24]. Studies have demonstrated changes in 5-HT levels and receptor expression in the gut mucosa of animal models with colitis, as well as in IBD patients, highlighting serotonin role in the pathogenesis of those diseases [25,26]. The disruption of serotonin signaling can exacerbate inflammation and contribute to worsening typical symptoms in these patients, such as diarrhea and abdominal pain, by affecting not only local gut functions but also immune cell activity and inflammatory responses [27].

Given these considerations, the potential of bLf to influence the intestinal serotonergic system represents a promising avenue for its functional modulation, which could benefit patients with intestinal inflammatory conditions. Therefore, the aim of this work was to study the effects of bLf on the intestinal serotonergic system both *in vitro* and *in vivo*. Specifically, we investigated bLf role using the Caco-2/TC7 cell line and examined its effects on the ileum of mice. Furthermore, we explored the protective role of bLf in a murine model of DSS-induced colitis.

2. Materials and methods

2.1. Bovine lactoferrin solutions preparation

Native bovine lactoferrin (bLf) was provided by the Tatua Nutritional Company (Morrinsville, New Zealand). The iron-saturation of this bLf was found to be less than 10%. The purity of the bLf was determined to be greater than 90%, as confirmed by SDS-PAGE analysis, which revealed a single band corresponding to a protein of approximately 80kDa. A concentrated stock solution of bLf was prepared in ultrapure water for cell-based assays and saline for *in vitro* and *in vivo* assays. The solutions were sterilized using a low-binding protein filter of 0.22μm. Its concentration was determined from its molecular extinction coefficient ($E^{1\%}_{280}$=1.27ml/cm/g). The concentration of bLf solutions used in the different assays was calibrated with reference to the concentration of the stock solution.

2.2. Cell culture

Caco-2/TC7 cells has been used in this study because they are an excellent human enterocyte-like model to study intestinal epithelial physiology [28–30]. The cells were cultured at 37°C in an atmosphere of 5% CO_2 and maintained in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 2mM glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 1% nonessential amino acids and 20% heat-inactivated foetal bovine serum (FBS) from Life Technologies (Carlsbad, CA, USA). The cells were passaged enzymatically (0.25% trypsin – 1mM EDTA) and sub-cultured in 25 or 75 cm^2 plastic culture flasks from Sarstedt (Nuembrecht, Germany). The medium was changed 72h after seeding and then every 48h. The experiments were conducted in cells cultured for 15 days when morphological and functional enterocyte-like differentiation had been established. The cells were treated with FBS-free culture medium containing bLf at different concentrations and periods. In long-term experiments, the medium and bLf were renewed daily. Prior to the experiments, the cell monolayer was analyzed, and none of the different conditions seemed to affect the morphology, proliferation, or monolayer integrity of the Caco-2/TC7 cells.

2.3. 5-HT uptake studies

Uptake measurements were carried out in cell cultures in 24-well plates seeded at a density of 4×10^4 cells/well, as previously described [31], either under a control condition and after different experimental conditions. The transport medium composition in mM, was: 137 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 10 HEPES pH 7.4, 4 glutamine, 1 ascorbic acid, 0.1% BSA, and both 0.2μM 5-HT and [^{3}H]−5-HT (1.5 μCi/ml) as the substrate. Before uptake studies, cells were preincubated at 37°C in an atmosphere of 5% CO_2 with substrate-free transport medium for 30min. The cells were washed with the substrate free transport medium at 37°C and then, incubated with the transport medium at 37°C for 6min. The uptake was stopped by removing the transport medium and washing the cells twice with an ice-cold transport medium containing 20μM 5-HT. Finally, cells were solubilized in 0.5ml of 0.1N NaOH and a sample of 200μL was taken for radioactivity counting (Wallac Liquid Scintillation Counter, Perkin-Elmer). The protein concentration was calculated using the kit Pierce BCA Protein Assay from Thermo Fisher Scientific (Waltham, MA, USA) with BSA as the standard. All sample results were calculated in pmol 5-HT/mg protein being and expressed as a percentage of the control value (100%). In the kinetic study, 5-HT uptake was measured in the concentration range 0.05–5μM, and the kinetic constants V_{max} and K_t were calculated.

2.4. Mice models

Male C57BL/6 mice (6–12 weeks) were purchased from Janvier Labs (Le Genest St. Isle, France) and allowed to acclimate for 1 week prior to the experiments. For the nonpathological model, the animals were kept in a conventional laboratory animal facility at the University of Zaragoza, housed in plastic cages with 5 mice per cage, under normal laboratory conditions (20±2°C, 40–60% relative humidity, and a 12-h light/dark cycle) with free access to standard chow and water. For the DSS-induced colitis model, housing and maintenance of the animals were carried out at the Centro de Investigación Biomédica de Aragón (CIBA), in compliance with previously described regulations and environmental conditions. All procedures were conducted under Project Licence PI40/17 and approved by the Ethics Committee for Animal Experiments of the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. All animals were euthanized via cervical dislocation, and ileum samples were removed and cleaned in an ice-cold solution of NaCl (0.9%). Intestinal samples for RNA studies were collected in RNAlater from Qiagen (Hilden, Germany) and stored for one day at 4°C, then subsequently frozen at −80°C.

Nonpathological model mice were randomly divided into two groups (n=5 per group): 1) control and 2) bLf. Control group daily received oral administration of 200 μl of NaCl 0.9% and mice from bLf group were treated for 10 days orally by gastric gavage with 35mg of bLf [34] diluted in 200 μl of saline solution. DSS-induced colitis mice were randomly divided into four groups (n=4 per group): (1) control, (2) DSS, (3) bLf, and (4) bLf+DSS. Over 8 days, all groups received 100 μl of NaCl 0.9% orally by gastric gavage, except the bLf and bLf+DSS groups, which received 12.5mg/mouse of bLf [32] in the same volume of saline solution. An injury-repair model of ulcerative colitis was employed [33]. On day 4, colitis was induced in the DSS and bLf+DSS groups by switching their drinking water to a 2.5% (w/v) solution of DSS (MW: 40,000Da, Panreac, Lörrach, Germany) for 5 days. To evaluate bLf protective effect against DSS-induced colitis in C57BL/6 mice, treatment with bLf commenced 3 days prior to DSS administration and continued once daily.

2.5. Disease activity index

The disease activity index (DAI) was used to evaluate the symptomatology of DSS-induced colitis. For this purpose, three parameters (weight loss, stool consistency, and presence of blood in stool) were evaluated daily from day 5 to day 9, corresponding to 24-hour intervals following the first DSS administration (day 4) and continuing through the final day of treatment (day 8). The data presented in the graph correspond to the last two days of evaluation (days 8 and 9), where the effects were more pronounced. The percentage of weight loss with respect to the control day was made, assigning 0 if the weight loss was <1%, 1 if it was between 1 and 5%, 2 if it was between 5 and 10%, 3 if it was between 10 and 15% and 4 if it was ≥15%. Stool consistency was determined as normal (0), soft (1) or presented diarrhoea (4). The investigator determining colon histological scores was blinded to the experimental group. Finally, the presence of blood in stool (resulting from epithelial damage and erosion produced by DSS) was evaluated using the Tri Slider FOB Guayaco kit (Sanilabo, Valencia, Spain) and scoring as no blood (0), occult blood (2), or visible blood (4).

2.6. Study of macroscopic colon damage

The colon was isolated, and its length was measured. Then, it was cut open longitudinally and washed with 0.9% saline solution. Macroscopic colitis signs were graded according to a standard scoring system: number of adhesions (0: 0, 1: 1, 2: 2, 3: >2), diarrhoea (0: normal, 1: loose stool, 4: water diarrhoea), tumefaction (0: absence, 1: moderate, 2: severe), stenosis (0: 0, 1: 1, 2: 2, 3: >2), mucus (0: absence, 1: presence), haemorrhage (0: absence, 1: manifest bleeding), erythema (0: no macroscopic changes, 1: <1cm, 2: ≥1cm), ulcers or erosions (0: 0, 1: <1cm, 2: ≥1cm) and occult blood (0: no, 1: yes). A total macroscopic damage score was calculated for each animal.

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

Caco-2/TC7 cells were cultured in 6-well plates seeded at a density of 2×10^5 cells/well and total RNA was extracted using TRI Reagent from Sigma-Aldrich (Saint Louis, MO, USA), following the manufacturer's protocol. For intestinal RNA extraction, the samples were first homogenized using the Ultra Turrax T25 from IKA (Staufen, Germany). The extracted RNA (1μg) was used as a template for first-strand cDNA synthesis using the qScript cDNA SuperMix from Quantabio (Beverly, MA, USA). The cDNAs obtained by reverse transcription (RT) were used to determine the mRNA level by real-time PCR using Fast SYBR Green Master Mix from Thermo Fisher Scientific (Waltham, MA, USA). Quantification of SERT, TPH1, TPH2, 5-HTR (1A, 2A, 2B, 3, 4 and 7) in Caco-2/TC7 cells and intestinal mice samples was performed using the Step One Plus Real-Time PCR System from Applied Biosystems (Foster City, CA, USA), with GAPDH and HPRT1 as housekeeping. The specific primers used are detailed in Table 1. The fluorescence raw data was analysed by the Applied Biosystems Step One Software v2.3 from Applied Biosystems. Thus, the mRNA relative expression was calculated as $\Delta\Delta Ct = \Delta Ct_{\text{control}} - \Delta Ct_{\text{treatment}}$ being $\Delta Ct = Ct_{\text{gene}} - Ct_{\text{calibrator}}$. GAPDH and HPRT1 were used as calibrators. Finally, the levels of relative gene expression were converted and expressed as fold difference ($=2^{-\Delta\Delta Ct}$).

2.8. Statistical analyses

The results were expressed as the mean ± the standard error of the mean (SEM). Kinetic study of the 5-HT transport values was performed by nonlinear regression, fitting the results to an equation containing a saturable (Michaelis-Menten) plus a non-saturable (diffusion) component. Differences between groups were statistically analyzed using the computer-assisted Prism GraphPad Program (Prism version 8.01, GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) followed by Bonferroni's (when parametric distribution is observed), Kruskal–Wallis tests (nonparametric) or unpaired t-tests were used to detect differences. Previously, normal distribution was confirmed with the Kolmogorov-Smirnov test. Significance level was set to $P < .05$.

3. Results

3.1. Effect of lactoferrin on SERT activity and mRNA expression in Caco-2/TC7 cells

To investigate the impact of bLf on SERT activity in an *in vitro* enterocyte model of intestinal epithelium, we examined 5-HT uptake in Caco-2/TC7 cells treated with different concentrations of bLf (range from 0.5mg/ml to 10mg/ml) for 24h. As depicted in Fig. 1A, bLf treatment resulted in a significant increase in 5-HT

Table 1
Primer sequences used for real-time PCR analysis of human cells (h) and intestinal mice (m) samples

Gen	Forward (5′−3′) and reverse (3′−5′) primers
hSERT	GGCCTGGAAGGTGTGATCA // GCGCTTGGCCCAGATGT
h5-HTR$_{1A}$	AACAACAACACATCACCACCGGC // AGATGCTCCATGGCGGGTGT
h5-HTR$_{2A}$	CAACTACGAACTCCCTAATG // AAACAGGAAGAAGACGATGC
h5-HTR$_{2B}$	GAATCACAGAAAAACAGCAAATGG // CCCATTTCGAATTCCATGTT
h5-HTR$_{3}$	GCCCTACTTTCGGGAGTTCAGCAG // TCTTGGTGGCTTGGGAGGTG
h5-HTR$_{4}$	CCTGTAATGGACAACTTGA // CCAATGTTATTCCAGCCTTG
h5-HTR$_{7}$	AGAGAAGCCAGACGGAGAGAA // TACGGCAGAGTCGAGAAAGTG
hGAPDH	CATGACCACAGTCCATGCCATCACT//TGAGGTCCACCACCCTGTTGCTGTA
hHPRT1	CTGACCTGCTGGATTACA // GCGACCTTGACCATCTTT
mSERT	GGCAACATCTGGCGTTTTCC // ATTTCGGTGGTACTGGCCCA
mTPH1	CACGAGTGCAAGCCAAGGTTT // AGTTTCCAGCCCCGACATCAG
mTPH2	GAGTTGCTCCACGCTTTGC // ACACTCAGTCTACATCCATCCC
m5-HTR$_{1A}$	TCTGTGAGAGCAGTTGCCACAT // AGCGGCAGAACTTGCACTTGAT
m5-HTR$_{2A}$	TGCCGTCTGGATTTACCTGGATGT // TACGGATATGGCAGTCCACACCAT
m5-HTR$_{2B}$	AGGAAATGAAGCAGACTGTGGAGG // CAGTGCAACAGCCAGAATCACAAG
m5-HTR$_{3}$	TCTTGCTGCCCAGTATCTTCCTCA // TTATGCACCAGCCGCACAATGAAG
m5-HTR$_{4}$	AATGCAAGGCTGGAACAACATCGG // TGTATCTGCTGGGCATGCTCCTTA
m5-HTR$_{7}$	TCTTCGGATGGGCTCAGAATGT // AACTTGTGTTTGGCTGCGCT
mGAPDH	AACGACCCCTTCATTGAC // TCCACGACATACTCAGCAC
mHPRT1	CTGGTGAAAAGGACCTCTCGAA // CTGAAGTACTCATTATAGTCAAGGGCAT

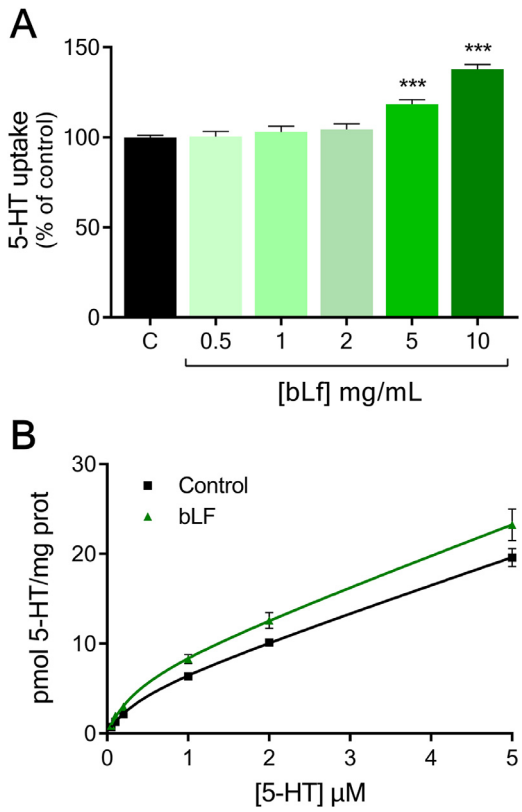

Fig. 1. Effect of bovine lactoferrin on 5-HT uptake in Caco-2/TC7 cells. A. Cells were treated with different concentrations of bLf (0.5, 1, 2, 5 y 10mg/ml) for 24h. 5-HT uptake was measured after 6min incubation with 0.2µM 5-HT. The results are expressed as the percentage of the control uptake (100%) and are the mean ± SEM of four replicates in eight independent experiments. Data was analysed combined by one-way ANOVA with Bonferroni's comparison test. $^{***}P < .001$ compared with the control (untreated cells). B. Kinetic study of SERT activity after treatment with bLf. The cells were treated during 24h with 10mg/ml bLf. The 5-HT range concentration was 0.05-5µM. The results are the mean of four experiments.

Table 2
Kinetic constants of 5-HT uptake in Caco-2/TC7 cells after lactoferrin treatment for 24h

	V_{max} (pmol 5-HT/mg protein)	K_t (µM)
Control	3.87±0.54	0.35±0.07
bLf (10mg/mL)	5.71±0.29*	0.34±0.07

The results are the mean ± SEM of four experiments. Data was analysed by t-test. $^{*}P < .05$ compared with control (untreated cells).

uptake at concentrations of 5mg/ml and 10mg/ml. Notably, the highest concentration of bLf (10mg/ml) induced a substantial 36% increase in 5-HT uptake, prompting its selection for subsequent experiments. To characterize the bLf effect on SERT activity, the kinetic study of the 5-HT transport was carried out (Fig. 1B), and kinetic constants V_{max} and K_t, which indicate the capacity and affinity of SERT respectively, were calculated (Table 2). 5-HT uptake was measured at different 5-HT concentrations (ranged 0.05-5µM) in Caco-2/TC7 cells treated with bLf 10mg/ml for 1 day. The results have shown that the treatment of cells with bLf increased 5-HT uptake by significantly affecting V_{max} (SERT capacity). However, bLf treatment did not seem to alter transport affinity (K_t) (Table 2).

To further elucidate and corroborate the effects of bLf on SERT, we assessed SERT mRNA expression in Caco-2/TC7 cells treated with 10mg/ml bLf for 24 hours and 10 days. As illustrated in Fig. 2, bLf significantly enhanced SERT mRNA expression. At 24-hours treatment, bLf induced approximately a 2-fold increase in SERT mRNA expression (Fig. 2A), whereas prolonged exposure (10 days) still elicited a substantial 1.5-fold increase (Fig. 2B).

3.2. Effect of lactoferrin on the mRNA expression of 5-HT receptors in Caco-2/TC7 cells

As lactoferrin modulates SERT mRNA expression and activity in Caco-2/TC7 cells, we investigated whether this protein could also affect other components of the intestinal serotonergic sys-

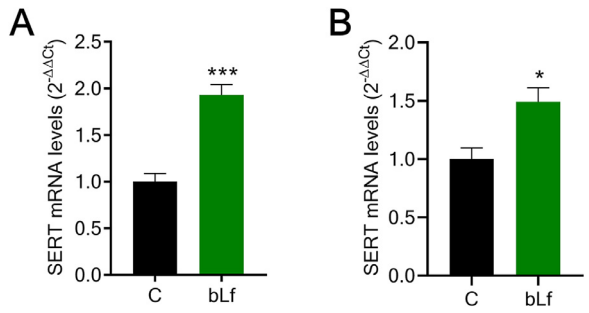

Fig. 2. Effect of bovine lactoferrin on SERT mRNA expression in Caco-2/TC7 cells. Cells were treated with bLf 10mg/ml for 24h (A) and 10 days (B). Relative mRNA quantification was performed in triplicate using the comparative Ct method ($2^{-\Delta\Delta Ct}$) normalized by HPRT1 and GAPDH mean. Results are expressed as arbitrary units (control=1) and are the mean±SEM of three replicates in four independent experiments. Data was analysed combined by unpaired t-test. $^{*}P < .05$ and $^{***}P < .001$ compared with the control (untreated cells).

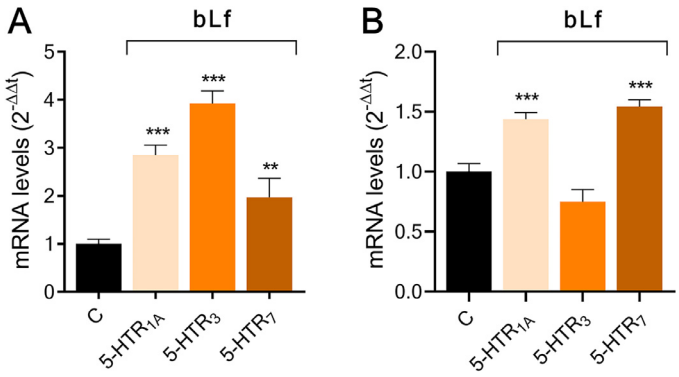

Fig. 3. Effect of bovine lactoferrin on the mRNA expression of 5-HTR_{1A}, 5-HTR_3 and 5-HTR_7 in Caco-2/TC7 cells. Cells were treated with bLf 10mg/ml for 24h (A) and 10 days (B). 5-HTRs mRNA expression was measured by qPCR. Relative quantification was performed in triplicate with the comparative Ct method ($2^{-\Delta\Delta Ct}$) normalized by HPRT1 and GAPDH mean. Results are expressed as arbitrary units (control=1) and are the mean ± SEM of three replications in four independent experiments. Data was analysed combined by one-way ANOVA with Bonferroni's comparison test. $^{**}P < .01$ and $^{***}P < .001$ compared with the control (untreated cells).

tem. Specifically, we examined the mRNA expression of well-characterized 5-HT receptors at the gastrointestinal level. Caco-2/TC7 cells were treated with bLf at 10mg/ml for 24h, and 10 days, and mRNA expression was determined by qPCR. Our analysis revealed that the mRNA expression of 5-HTR_{2A}, 5-HTR_{2B}, and 5-HTR_4 was undetectable in Caco-2/TC7 cells, indicating their absence in this cell line. Lactoferrin significantly influenced the mRNA expression of 5-HTR_{1A}, 5-HTR_3, and 5-HTR_7 (Fig. 3). Specifically, the expression of 5-HTR_{1A} and 5-HTR_7 was consistently increased at 24h and 10 days. Elsewhere, 5-HTR_3 mRNA expression was enhanced after 24 hours of bLf treatment, whereas its expression decreased following prolonged exposure to lactoferrin (10 days).

3.3. Effect of lactoferrin on serotonergic system mRNA expression in the mice ileum

After having proven the lactoferrin effect on the intestinal serotonergic system in enterocyte-like Caco-2/TC7 *in vitro* model, we sought to validate these findings in an *in vivo* mice model. Therefore, we analysed the expression of different components of intestinal serotonergic system in the ileum of mice. mRNA expression of SERT, TPH enzymes (TPH1 and TPH2), and 5-HT receptors (1A, 2A, 2B, 3, 4, and 7) were analysed in the ileum from mice treated with 35mg of bLf over a period of 10 days. As shown in Fig. 4A, lactoferrin significantly increased SERT mRNA expression in ileum samples, more than two-fold compared to the control, which aligns with the observations in the *in vitro* model. Additionally, TPH2 expression was significantly elevated, while TPH1 expression remained unchanged (Fig. 4A). Consistent with our earlier findings, mRNA expression of all 5-HT receptors (1A, 2A, 2B, 3, 4, and 7) was increased in the ileum of bLf-treated mice, with particularly significant increases observed for receptors 2A and 7. compared to control mice (Fig. 4B).

3.4. Lactoferrin attenuates colon damage in a murine model of DSS-induced colitis

After corroborating the results of *in vitro* lactoferrin in the ileum of mice, we investigated its potential to mitigate macroscopic colon damage in a DSS-induced colitis model. An injury-repair model of colitis was induced by administering 2.5% DSS in the drinking water for 5 days. Mice treated with DSS were additionally administered daily with 12.5mg of bovine lactoferrin for 8 days, starting three days before DSS treatment, and continuing bLF administration for the 5 days of DSS treatment. Colitis was characterized by weight loss, presence of blood in faeces, and changes in stool consistency. As expected, the DSS treatment substantially increased the disease activity index (DAI). Lactoferrin pretreatment prevented the colon damage induced by DSS as shown in the decreased of DAI (Fig. 5A). In accordance, macroscopic evaluation revealed significant colon damage induced by DSS, which was partially alleviated by pretreatment with lactoferrin, indicating a significant protective effect against DSS-induced damage (Fig. 5B). Similarly, colon length was reduced in animals with DSS treatment, which was avoided by pretreatment with lactoferrin as shown in Fig. 5C. Administration of bLf to mice not exposed to DSS did not induce any macroscopic alterations, suggesting the nontoxic nature of bLf at the dosage used.

3.5. Lactoferrin reduces DSS-induced alterations of the serotonergic system in mice colon

The role of lactoferrin in serotonergic modulation was further investigated by assessing mRNA expression of SERT, TPH1, TPH2, and 5-HT receptors (1A, 2A, 2B, 3, 4, and 7) in both the proximal and distal colon of mice with DSS-induced colitis pretreated daily with 12.5mg of bLf. In contrast to the ileum, the expression of 5-HT_{2A} receptor was undetectable in both colon segments.

As depicted in Figs. 6 and 7, SERT mRNA levels decreased in the proximal and distal colon of DSS-treated mice compared to control. However, the reduction was more pronounced in the distal colon, where SERT mRNA levels were significantly decreased by 60% compared to the control. Lactoferrin did not alter SERT mRNA expression *per se*; however, it effectively prevented the DSS-induced decrease in SERT mRNA levels in both colon segments. Consistent with findings on SERT mRNA expression, DSS administration exerted a greater impact in the mRNA levels of TPH1, TPH2 and 5-HT receptors in distal colon, significantly increasing the expression levels of TPH1, TPH2, 5-HTR_{1A}, 5-HTR_{2B}, 5-HTR_3 and 5-HTR_7, while decreasing 5-HTR_4 expression (Fig. 7). Similar effects were observed in the proximal colon, where only the increase in 5-HTR_3 expression reached statistical significance (Fig. 6). Interestingly, bLf did not alter the baseline mRNA expression of serotonergic system components in colonic tissues. However, it significantly mitigated the DSS-induced upregulation of 5-HTR_{2B} and downregulation of 5-HTR_4 in both proximal (Fig. 6) and distal colon (Fig. 7). Furthermore, bLf significantly reduced DSS-induced alterations in the mRNA expression of all analysed genes of the serotonergic system, specifically in the distal colon (Fig. 7).

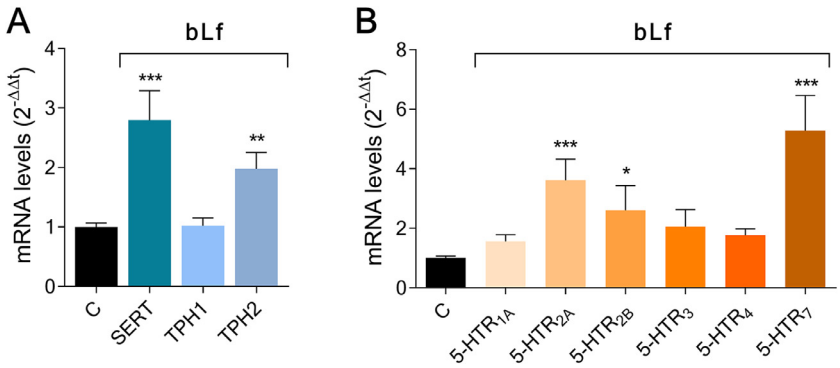

Fig. 4. Gene expression of the serotonergic system in ileum from mice treated with bovine lactoferrin. SERT, TPH enzymes (THP1 and TPH2) (A) and 5-HT receptors (1A, 2A, 2B, 3, 4 and 7) (B) mRNA expression was measured in ileum of control and bLf-treated mice by real-time PCR. Relative quantification was performed in triplicate with the comparative Ct method ($2^{-\Delta\Delta Ct}$) normalized by HPRT1 and GAPDH mean. Results are expressed as arbitrary units (control=1) and are the mean ± SEM of data from five animals. Data was analysed by one-way ANOVA with Bonferroni's comparison test. $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ compared with the control group.

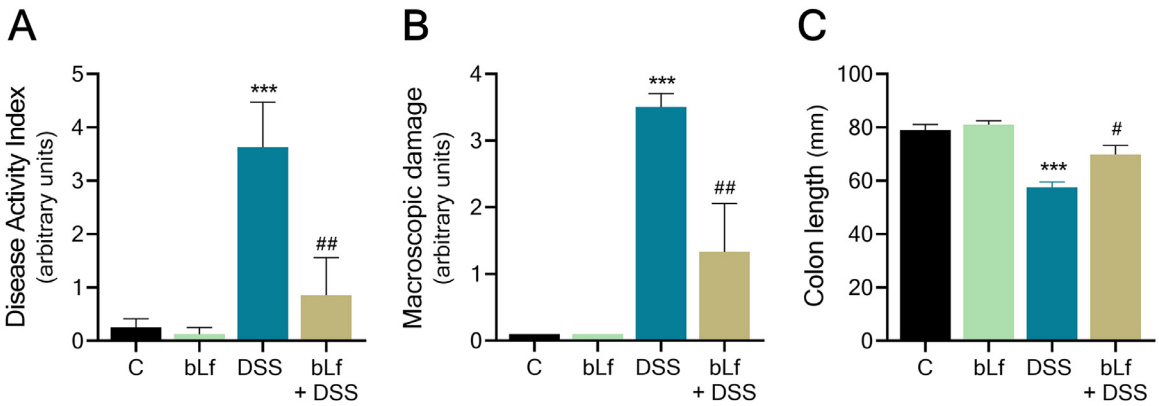

Fig. 5. Effect of bovine lactoferrin on DSS-induced colon damage. (A) Disease activity index (DAI) assessed on the last two days of treatment in all animal groups: C (control); bLf (treated with bLf); DSS (treated with DSS); and bLf+DSS (pretreated with bLf and then with DSS and bLf). (B) Macroscopic damage score calculated according to a standard scoring system. (C) Colon length (mm). Data are presented as mean±SEM of data from four animals. Data was analysed by one-way ANOVA with Bonferroni's comparison test. $^{***}P < .001$ compared with the control group; $^{\#}P < .05$ and $^{\#\#}P < .01$ compared with the DSS group.

4. Discussion

Our study demonstrates that bLf exerts significant modulatory effects on the intestinal serotonergic system, presenting potential protective benefits in the context of intestinal inflammation. bLf treatment led to increased activity and enhanced mRNA expression of SERT and various serotonin receptors at both short and long treatment periods in the Caco-2 intestinal epithelial cell model. However, the mRNA expression of the 5-HTR_3 receptor decreased after 10 days of treatment, suggesting a complex regulatory role of bLf over prolonged periods. The increase in SERT activity and mRNA expression could be driven by an augmentation of SERT transporter in the cell membrane, as evidenced by the significant rise in both Vmax (capacity) after 24h of treatment. In parallel with our *in vitro* findings, bLf administration in a nonpathological murine model over 10 days showed a notable upregulation in 5-HTRs, TPH2 enzyme, and SERT mRNA expression in the ileum. Importantly, this was achieved without observable adverse effects on body weight, faecal blood presence, or diarrhoea, underscoring bLf's gastrointestinal safety profile [34]. The observed potent short-term modulatory effects of bLf on the serotonergic system *in vitro* may stem from a rapid and effective initial cellular response, which triggers widespread mRNA expression of SERT and 5-HT receptors. However, as treatment duration progresses, cells may adapt or exhibit reduced responsiveness, possibly due to tolerance mechanisms and negative feedback aimed at maintaining homeostasis. Differential receptor mRNA expression patterns reflect specific signaling pathways or molecular interactions between bLf and distinct receptors over intermediate to prolonged periods. This supports the hypothesis of cellular adaptation dynamics in response to bLf, where certain receptors may become more or less sensitive or efficient in signal transduction over time.

These results collectively indicate that bLf plays a regulatory role in serotonin signaling, potentially reducing extracellular 5-HT levels through increased SERT activity and expression and modulating 5-HTRs mRNA levels. Such modulation is crucial for maintaining intestinal serotonin homeostasis, essential for key digestive functions like motility and nutrient absorption, thus promoting intestinal health [35]. Furthermore, the increase in TPH2 mRNA expression produced by bLf in the ileum suggests enhanced neuronal 5-HT production, which typically correlates with protective effects compared to mucosal serotonin [36,37]. Consistent with our findings, previous studies have highlighted the role of bLf in serotonergic system regulation by modulating 5-HT biosynthesis, particularly in the nematode *Caenorhabditis elegans*, through cat-4 gene overexpression. This gen is orthologous to human GTP cyclohydrolase I, which is involved in the synthesis of tetrahydrobiopterin (BH4, THB), crucial for 5-HT biosynthesis [38]. Additionally, various researchers emphasize that both bLf and some of its derived peptides regulate 5-HT biosynthesis indirectly by exerting a bacteriostatic effect on certain gut bacteria related to 5-HT synthesis. *In vitro* studies confirm the bacteriostatic role of lactoferrampin on

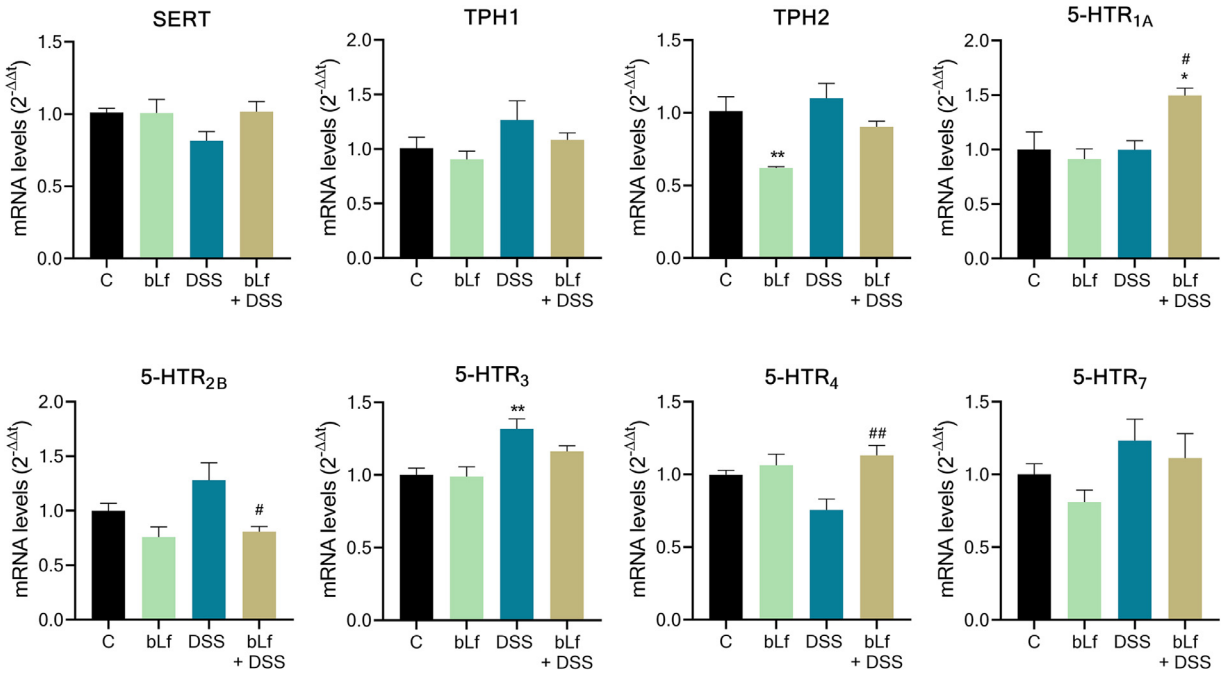

Fig. 6. Effect of bovine lactoferrin on serotonergic system alterations induced by DSS in proximal colon. SERT, THP1, TPH2 and 5-HT receptors (1A, 2B, 3, 4 and 7) mRNA expression was measured by qPCR in the proximal colon of mice from four groups: control, DSS, bLf and bLf+DSS. Relative quantification was performed in triplicate with the comparative Ct method ($2^{-\Delta\Delta Ct}$) normalized by HPRT1 and GAPDH mean. Results are expressed as arbitrary units (control=1) and are the mean±SEM of data from four animals. Data was analysed by one-way ANOVA with Bonferroni's comparison test. $^{*}P < .05$ and $^{**}P < .01$ compared with the control group; $^{\#}P < .05$ and $^{\#\#}P < .01$ compared with the DSS group.

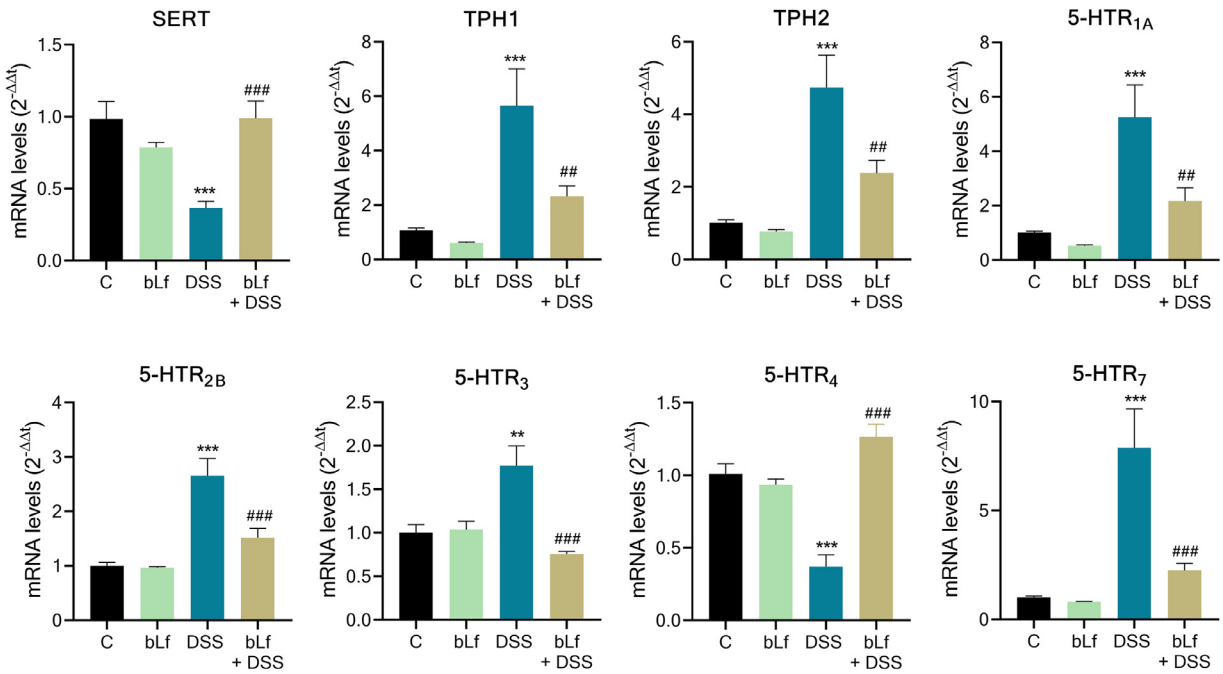

Fig. 7. Effect of bovine lactoferrin on serotonergic system alterations induced by DSS in distal colon. SERT, THP1, TPH2 and 5-HT receptors (1A, 2B, 3, 4 and 7) mRNA expression was measured by qPCR in the distal colon of mice from four groups: control, DSS, bLf and bLf+DSS. Relative quantification was performed in triplicate with the comparative Ct method ($2^{-\Delta\Delta Ct}$) normalized by HPRT1 and GAPDH mean. Results are expressed as arbitrary units (control=1) and are the mean ± SEM of data from four animals. Data was analysed by one-way ANOVA with Bonferroni's comparison test. $^{**}P < .01$ and $^{***}P < .001$ compared with the control group; $^{\#\#}P < .01$ and $^{\#\#\#}P < .001$ compared with the DSS group.

Escherichia coli, which is capable of synthesizing 5-HT *per se*. Similarly, it has been demonstrated that oral administration of lactoferrin in mice exerts a comparable effect on intestinal *Clostridium ramosum*, which induces 5-HT synthesis by enterochromaffin cells through the production of SCFAs [39]. Moreover, several studies have highlighted lactoferrin's role in modulating the immune system through pattern recognition receptors (PRRs), which are involved in recognizing microbial components and initiating immune responses [30,34,40]. These receptors, in addition to their specific functions, have also been implicated in influencing the regulation of the serotonergic system in various contexts [22]. This suggests that lactoferrin influence on PRRs could potentially contribute to its broader regulatory effects on the serotonergic system.

In inflammatory contexts compromising intestinal homeostasis, bLf ability to modulate serotonergic signaling could offer potential therapeutic benefits. Serotonin not only influences gastrointestinal function but also contributes to inflammation. The capacity of bLf to modulate serotonergic system could provide anti-inflammatory effects and contribute to the preservation of gastrointestinal tract integrity. This regulation may also help counteract gastrointestinal system disturbances associated with inflammation, including not only motility disorders but also changes in fluid secretion and pain perception, among other issues [27]. Similar effects to bLf have been observed in Caco-2/TC7 cells treated with other anti-inflammatory substances like IL-10, enhancing SERT activity and expression in the short term, thereby reducing intestinal tissue serotonin levels [41].

Moving from physiological conditions to the inflammatory context of IBD, our study sought to discern bLf modulation of the serotonergic system under pathological conditions. This approach is crucial for understanding its therapeutic potential in IBD and addressing associated clinical challenges. The findings from our study revealed several notable effects of bLf on the serotonergic system and other relevant aspects within the inflammatory context of colitis. bLf mitigated colon damage, reinforcing its protective effects in colitis. Other studies have also observed symptomatic improvements in colitis models treated with lactoferrin, further bolstering evidence of its efficacy as a therapeutic agent in such pathologies [42,43].

Detailed analysis of the proximal and distal colon in DSS-induced ulcerative colitis mice revealed significant modulation in the serotonergic system mRNA expression by bLf. As might be expected, DSS administration decreased SERT mRNA while increasing serotonin synthesis enzymes and serotonin receptors, except for 5-HTR_4, enhancing both 5-HT availability and serotonergic signaling. The effect of DSS was generally more pronounced and significant in the distal colon compared to the proximal colon. This suggests that this region of the intestine may be particularly relevant in the pathogenesis of colitis, at least as far as this inflammatory model is concerned, and in the response to potential treatments, consistent with previous findings documenting higher levels of pro-inflammatory mediators and greater histopathological damage due to DSS in the distal colon [44]. This fact highlights the need to understand regional differences in intestinal response not only to inflammatory agents but also to substances evaluated for their therapeutic effects. bLf pretreatment attenuated these effects in both colon segments, counteracting SERT reduction and mitigating DSS impact on enzyme and receptors mRNA expression. The ability of bLf ability to mitigate these changes at molecular level could be associated with its protective effect in DSS-induced colitis. Various studies in both animal models and humans have demonstrated changes in the expression and functionality of one or more elements of 5-HT signaling in the context of IBD [27], therefore, lactoferrin could play a crucial role in maintaining serotonergic homeostasis. Consequently, lactoferrin may modulate the inflammatory response in IBD patients, and the associated symptoms, by reducing the impact of inflammation on the 5-HT signaling. The precise site of action of bovine lactoferrin remain to be fully elucidated. Regarding the nature of the active component, it is likely that the observed effects result from a combination of mechanisms. While intact lactoferrin may exert direct effects on epithelial and immune cells via receptor-mediated pathways, it is also known that lactoferrin can be partially digested into bioactive peptides, such as lactoferricin, which may contribute to its biological activity [45]. Additionally, lactoferrin has been shown to induce host responses, including the modulation of cytokine production and immune cell activation, which could mediate some of the downstream effects observed [46].

Serotonin acts as a pro-inflammatory mediator, exacerbating responses in various tissues including the gastrointestinal tract. Elevated 5-HT levels contribute to excessive immune cell activation, as well as motor, sensory, and secretory dysfunctions, worsening symptoms in IBD and Irritable Bowel Syndrome (IBS) patients [47]. Studies also link increased 5-HT levels during DSS-induced colitis to heightened inflammation in the colon through mechanisms like upregulated matrix metalloproteinases MMP-3 and MMP-9 [48], and inhibited β-defensin production by colon epithelial cells [49]. Therefore, the observed decrease in SERT mRNA and increase in synthesis enzymes in our study likely elevate 5-HT levels, exacerbating inflammation. Our findings align with research in other murine colitis models, where increased 5-HT content was observed following DSS treatment, accompanied by an increase in 5-HT and TPH1 immunopositive cells and reduced SERT expression [50]. IFN-γ and TNF-α may negatively regulate SERT in colitis, as evidenced by reduced transporter function and expression observed in Caco-2 cells exposed to pro-inflammatory cytokines [51]. Furthermore, recent research highlights that an increase in TPH1 expression not only elevates 5-HT levels but may also be associated with the severity of colitis by inducing changes in the intestinal microbiota. It was observed that the microbiota from $TPH1^{-/-}$ mice showed protective effects against colitis compared to microbiota from $TPH1^{+/+}$ mice [49]. Conversely, the upregulation of the enzyme TPH2 under inflammatory conditions could be interpreted as a compensatory mechanism to protect the enteric nervous system and intestinal mucosa from the adverse effects associated with DSS-induced inflammation [36,37].

In addition, the upregulation of serotonergic receptors in colitic mice, as observed in our model, further underscores the role of altered serotonin signaling in the progression of colitis. Elevated receptor levels suggest enhanced sensitivity to serotonin, potentially intensifying gastrointestinal symptoms and driving the inflammation associated with the disease. This heightened sensitivity may disrupt the normal regulation of intestinal functions, mucosal integrity, and immune responses, thereby compounding disease severity. Among the serotonergic receptors, 5-HT_7 stands out as the most significantly upregulated in the distal colon in our study. This receptor has been increasingly recognized for its role in promoting intestinal inflammation. Inhibition of 5-HT_7 signaling has been shown to reduce inflammation, histopathological damage, and proinflammatory cytokine levels in experimental colitis models, positioning it as a promising therapeutic target [52,53]. In our study, the restoration of 5-HT_7 receptor mRNA level following bLf treatment highlights its potential as a key regulator of gut inflammation and underscores its relevance in modulating serotonin signaling in IBD. Conversely, the reduced mRNA level of the 5-HTR_4 receptor in our DSS-induced colitis model could exacerbate disease pathology. This receptor is critical for maintaining intestinal motility in healthy mice and is thought to exert anti-inflammatory effects in colitis [54]. Its downregulation may impair motility, worsen inflammation, and further destabilize intestinal homeosta-

sis. Importantly, the fact that bLf treatment attenuates the DSS-induced reduction of 5-HTR_4 mRNA in both colon segments highlights the potential of lactoferrin in mitigating this aspect of colitis. By preserving the expression of this receptor, bLf may help maintain intestinal motility and counteract the inflammatory response, contributing to a more balanced intestinal environment. Together, these findings emphasize the dual role of serotonergic signaling in colitis, where upregulation of pro-inflammatory receptors like 5-HT_7 and downregulation of protective receptors like 5-HTR_4 contribute to disease progression. These insights provide a basis for exploring targeted therapeutic interventions aimed at restoring serotonergic balance in IBD, possibly through agents like lactoferrin.

The clinical relevance of our findings is underscored by studies in IBD patients, demonstrating similar serotonin system alterations. Decreased SERT expression, coupled with increased 5-HT levels and enterochromaffin cell density, has been observed in Crohn's disease and ulcerative colitis patients' intestinal mucosa [55,56]. Furthermore, increased TPH1 mRNA levels have been reported in Crohn's disease patients [55], alongside reduced SERT expression also in IBS patients and other inflammatory intestinal pathologies [57]. These alterations are believed to vary depending on factors such as disease severity or stage. Serotonergic receptors also exhibit altered expression in both animal models and patients with IBD. Therefore, modulation of the serotonergic system may be relevant in managing IBD and other gastrointestinal disorders [27]. The convergence of evidence across studies in animal models and humans underscores the importance of understanding the underlying mechanisms of serotonergic system regulation in intestinal inflammation, which could have therapeutic implications for treating IBD.

The dearth of specific research on bLf effect on serotonergic modulation in inflammatory contexts like IBD is evident. Nevertheless, our study highlights lactoferrin as a multifaceted molecule that integrally impacts this system, contrasting with other substances like probiotics (e.g., *Lactobacillus acidophilus*) and specific compounds (e.g., corylin), which have been shown to regulate the serotonergic system during colitis, primarily influencing 5-HT synthesis or specific receptors [58]. This broader impact suggests that bLf may offer additional benefits not covered by other treatments.

Beyond our findings, lactoferrin has shown promise as a beneficial dietary ingredient in both *in vivo* and *in vitro* models of intestinal inflammation and dysbiosis. It has been demonstrated to protect the intestinal barrier, modulate mucosal immunity, and reduce inflammatory markers. Specifically, lactoferrin can restore tight junction integrity [59,60], inhibit key inflammatory pathways [61], and alleviate disease severity in colitis models [42,59]. Additionally, it has been observed to positively influence gut microbiota and reduce the pro-inflammatory cytokines in a murine model of intestinal dysbiosis induced by clindamycin [34,62]. These effects complement our results and suggest that lactoferrin could be valuable for improving gut health. Furthermore, its well-documented safety and tolerability in humans, including infants and children, combined with its ease of production [15], underscores its viability for managing inflammatory conditions and promoting gastrointestinal well-being. In conclusion, bovine lactoferrin emerges from our study as a potent regulator of intestinal serotonergic system, offering protective benefits in a murine model of colitis. These findings reinforce its potential as a functional food ingredient for conditions like IBD, warranting further investigation into its applications for enhancing gut health through dietary interventions.

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Author statement

All authors have significantly contributed to the conception, design, and development of this manuscript and have approved the final version submitted to *The Journal of Nutritional Biochemistry*. The manuscript is original, has not been published previously, and is not under consideration for publication elsewhere. We declare no conflicts of interest. The study complies with the ethical standards of the journal and the relevant institutional and international guidelines.

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.

CRediT authorship contribution statement

Berta Buey: Writing – original draft, Investigation. **Inés Abad:** Investigation. **Andrea Bellés:** Investigation. **Marta Castro:** Methodology, Data curation. **Marta Sofía Valero:** Methodology, Data curation. **María Pilar Arruebo:** Methodology, Conceptualization. **Laura Grasa:** Funding acquisition, Conceptualization. **Lourdes Sánchez:** Writing – review & editing, Investigation, Funding acquisition. **Miguel Ángel Plaza:** Methodology, Conceptualization. **José Emilio Mesonero:** Writing – review & editing, Supervision, Project administration, Formal analysis. **Eva Latorre:** Writing – review & editing, Supervision, Formal analysis.

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