Lactoferrin modulates oxidative stress and inflammatory cytokines in a murine model of dysbiosis induced by clindamycin

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Abstract

Antibiotics, specifically clindamycin, cause intestinal dysbiosis, reducing the microbiota with anti-inflammatory properties. Furthermore, clindamycin can induce alterations in the immune responses and oxidative stress. Lactoferrin, among other activities, participates in the maintenance of intestinal homeostasis and reduces dysbiosis induced by antibiotic treatment. The aim of this study was to analyze the effect of native and iron-saturated bovine LF in a murine model of dysbiosis induced by clindamycin. Six groups of male C57BL/6 mice were treated with saline (control), clindamycin (Clin), native lactoferrin (nLF), iron-saturated lactoferrin (sLF), nLF/Clin or sLF/Clin. Oxidation caused in the intestinal cells of the ileum of animals subjected to different treatments was analyzed, focusing on lipid peroxidation and protein carbonyl content. The expression of inflammatory mediators was determined by qRT-PCR. Treatment with clindamycin did not modify lipid peroxidation, but significantly increased protein carbonyl levels up to almost 5-fold respect to the control, an effect that was reversed by orally administering sLF to mice. Furthermore, clindamycin increased the expression of interleukin-6 and TNF-α by 1- and 2-fold change, respectively. This effect was reversed by treatment with nLF and sLF, decreasing the expression to basal levels. In conclusion, this study indicates that lactoferrin can prevent some of the effects of clindamycin on intestinal cells and their associated immune system.

Keywords: antibiotics, interleukin-6, immune system, ROS, TNF-α.

Introduction

Antibiotics can lead to undesirable collateral effects, such as changes in intestinal microbiota, promoting dysbiosis and having an impact on the development of innate and adaptive immune responses against pathogens. Antibiotic-induced dysbiosis also increases the susceptibility to infections in later life (Shekhar & Petersen, 2020). Clindamycin is a lincosamide antibiotic whose action is based on slowing or stopping the growth of Gram-positive and anaerobic bacteria. This antibiotic is highly concentrated in the faeces, having a negative impact on the intestinal microbiota (Buffie et al., 2012). Clindamycin has been shown to induce dysbiosis, reducing the presence of certain microorganisms with anti-inflammatory properties (Bellés et al., 2022).

In addition, some bactericidal antibiotics can induce a common oxidative damage, generating variable levels of deleterious reactive oxygen species (ROS) and causing damage to DNA, proteins and lipids, resulting in cell death (Guillouzo & Guguen-Guillouzo, 2020). Clindamycin, in particular, causes oxidative damage to DNA and lipids (Xiao et al., 2019). At low concentrations, ROS act as important mediators in almost all stages of the inflammatory process. However, overproduction of ROS can cause cellular damage and promote chronic inflammation (Chelombitko, 2018).

Bovine lactoferrin (LF) is a cationic iron-binding glycoprotein present in exocrine secretions of mammals, such as milk, saliva, tears, etc. LF is secreted in its open form without iron (apo-LF), but it is able to bind two ferric ions giving rise to its closed form (holo-LF) (Adlerova, Bartoskova, & Faldyna, 2008). It has been shown that depending on LF iron saturation it can develop divergent effects on the growth of probiotic bacteria (Li et al., 2013; Fan et al., 2022). The increase of iron saturation in LF decreases its bacteriostatic capacity, with apo-LF being more functional (Orsi, 2004). However, holo-LF is not easily degraded and it is highly stable, especially when heated and stored for long time. For this reason, holo-LF is often used as an additional ingredient in some products (Fan et al., 2022). LF has numerous benefits at the intestinal level, such as to favour iron absorption, increase the intestinal maturation or its barrier function, exert antibacterial and antiviral activity, or modulate the gut microbiota (Conesa et al., 2023). Furthermore, LF has been reported to contribute to the maintenance of intestinal homeostasis and to counter dysbiosis induced by antibiotic treatment; in addition to inhibiting the inflammatory response (Bellés et al., 2022). In this regard, LF, as an anti-inflammatory agent, is capable of maintaining a physiological balance of ROS by chelating free iron, essential for the production of ROS, or regulating antioxidant enzymes, thus reducing the inflammatory response (Cutone et al., 2020; Conesa et al., 2023).

The most widely adopted way for LF administration is orally. However, LF does not pass intact through the stomach and is hydrolyzed during gastric digestion, releasing some bioactive peptides, such as lactoferricin and lactoferrampin. Therefore, although hydrolysis could affect the functional properties of LF, its degradation also could be beneficial for developing some activities (Wang et al., 2019). Furthermore, the susceptibility of LF to digestion depends on the matrix where it is found, being digested more easily when is in a liquid product than in a solid one. In addition, the degree of glycosylation of LF also affects its stability in the gastrointestinal tract (Wang et al., 2019).

The inflammatory response is highly regulated in the gastrointestinal tract, ensuring a balance of pro-inflammatory and anti-inflammatory cytokines (Schenk & Mueller, 2008). Intestinal epithelial cells respond to external stimuli by expressing inflammatory factors and chemokines through the corresponding signalling pathways. Furthermore, these cells can recruit white blood cells to kill damaged cells or foreign pathogens. Therefore, intestinal development in the first period of life is crucial for the health of infants and children (Fan et al., 2022). LF plays an important role in modulating the immune system. It is considered an anti-inflammatory protein that helps prevent and treat inflammatory bowel diseases (IBD). Its immunomodulatory effect is based on inhibiting the synthesis of pro-inflammatory cytokines, such as TNF-α and interleukin-6 (IL-6), and promoting the production of anti-inflammatory cytokines, such as IL-4 and IL-10 (Conesa et al., 2023).

Therefore, the main objective of this study was to evaluate the effect of native and iron-saturated LF on the oxidative stress and expression of inflammatory mediators in the ileum of mice with dysbiosis induced by the antibiotic clindamycin.

Material and methods

Sample preparation

Native bovine LF (iron saturation below 10%) used for this study was kindly donated by Tatua Nutritionals (Morrinsville, New Zealand). Its purity of 90% was checked by SDS-PAGE, showing a single band at 80 kDa corresponding to the protein. For stock solution preparation, native LF (nLF) was dissolved in saline at a concentration of 200 mg/mL and processed as detailed previously (Bellés et al., 2022). The LPS level of this LF was determined in a previous study (Abad et al., 2022) and it was considered minimal (8×10^{-3} endotoxin units per mg of protein), consequently, we assumed it did not influence the results obtained.

To prepare iron-saturated LF (sLF), the procedure described by Graikini et al. (2023) was followed. Briefly, an iron complex of 20 mM $FeCl_3$ and 80 mM sodium nitrile acetate (NTA)

(FeNTA) and 10 mM $NaHCO_3$ (1:1, v:v) was added to LF (3 µL per mg of protein) and the mixture maintained during 24 h at 4 °C. Afterwards, the protein was subjected to Sephadex G-25 chromatography to remove unbound iron. The resulting sLF was considered 100% iron saturated.

Both nLF and sLF were adjusted to a concentration of 175 mg/mL for their use in the animal experiments.

Animal model and treatments

The study was carried out with 30 male C57BL/6 mice of 8-12 weeks old (Janvier Labs, Le Genest-Saint-Isle, France). They were kept in a conventional laboratory animal facility at the University of Zaragoza at a range of temperature between 20-22 °C, under a cycle of 12 h light/dark, with free access to water and animal chow. For the experiment, mice were randomly divided into 6 groups (n = 5 per group): control (receiving saline orally by gastric gavage for 10 days and representing the negative control group), clindamycin (Clin), native bovine lactoferrin (nLF), iron-saturated bovine lactoferrin (sLF), native bovine lactoferrin + clindamycin (nLF/Clin) and iron-saturated bovine lactoferrin + clindamycin (sLF/Clin) (Figure 1). All groups tested were treated equally, housed in the same room, kept in the same cages and maintained by the same personnel.

During the experiment, mice treated with clindamycin were fed for 10 days with saline and on day 4 received a single intraperitoneal (IP) injection of 200 µg of clindamycin (Normon Laboratories, Madrid, Spain) diluted in 0.2 mL of saline. This *in vivo* experiment performed in the present study encompassed numerous analyses, such as the determination of intestinal microbiota composition and of the expression of TLR receptors (Bellés et al., 2022). Therefore, the dose of clindamycin used was chosen based on previous dysbiosis experiments carried out in mice (Buffie et al., 2012).

Mice of the groups nLF and sLF were treated for 10 days orally by gastric gavage with 35 mg of nLF or sLF, diluted in 0.2 mL of saline. Similar doses of LF have been shown to exert an effect on the cytokine responses and intestinal immune system of mice in previous studies (Wakabayashi et al., 2004a; Wakabayashi et al., 2006). Mice from nLF/Clin and sLF/Clin groups were fed for 10 days with 35 mg of nLF or sLF and on day 4 received an IP injection of 200 µg of clindamycin (Figure 1). The dose of 35 mg administered to the mice per day would be equivalent to about 140 mg/kg in humans (Nair & Jacob, 2016). Following each treatment, mice were humanely euthanized by cervical dislocation.

All procedures were conducted under Project Licence PI40/17 and approved by the in-house 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.

Histopathology

At postmortem, sections of the ileum, cecum and colon of each animal were collected for quantitative histopathological assessment of intestinal inflammation. Tissue sections were fixed in 10% neutral-buffered formalin for 48-72 h and routinely processed for paraffin embedding and hematoxylin-eosin staining. Several histopathological parameters were evaluated in the three sections of the intestine: number of polymorphonuclear (PMN) cells, number of globet cells, integrity of intestinal epithelial barrier and number of bacteria in intestinal crypts. The selected parameter for evaluation was the number of PMN cells as it was the only one with significant differences between the group treated with clindamycin and the control group. The total number of PMN cell in the intestinal lamina propria was quantified in 10 high-power fields (x400 magnification) by two pathologists blinded to the treatment groups.

Assessment of oxidative stress

Ileums were collected, washed with 0.9% NaCl and stored at −80 °C until processed. Tissue samples were homogenized in cold 50 mM Tris buffer, pH 7.4, using Yellowline DI25 Basic Ultra-Turrax (IKA-Werke, Staufen Germany). The homogenate was centrifuged at 3000 g for 10 min at 4 °C. The supernatant was collected for lipid peroxidation and protein carbonyl analysis.

The level of lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) as previously described (Gonzalo et al., 2011). In short, MDA + 4-HDA reacts with 1-methyl-2-phenylindole, giving rise a stable chromophore, whose absorbance can be measured spectrophotometrically at 586 nm. The samples were analyzed in duplicate. Lipid peroxidation was calculated in nmol MDA + 4-HDA per mg of protein. Protein concentration was determined by the Bradford method (Bio-Rad, Madrid, Spain). Results of lipid peroxidation were expressed as the percentage respect to the control value (100%).

The level of protein oxidation was analyzed by determining protein carbonyl content as previously described (Gonzalo et al., 2011). Tissue homogenates were incubated with 2,4-dinitrophenylhydrazine (DNPH), a carbonyl reagent, at 37 °C for 1 h and protein carbonylation was measured at 375 nm. The samples were analyzed in triplicate. Results were calculated in

nmol carbonyl groups per mg of protein and expressed as the percentage respect to the control value (100%).

RNA extraction and RT-PCR

Ileum samples were collected and preserved for 24 h in RNAlater solution (Ambion, Thermo Fisher Scientific, Madrid, Spain) and then, they were stored at -80 ºC until analysis. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). A fragment of each sample was taken and placed in a Precellys® tube (Precellys® Ceramic kit, Bertin Instruments, Montigny-le-Bretonneux, France) for homogenization. 600 µL of RLT lysis buffer supplemented with β-mercaptoethanol were added in a 100:1 (v/v) ratio. The samples were introduced into a Precellys®-24 homogenizer (Bertin Instruments) and processed with a program of 2 cycles of 20 s at 5000 rpm with a waiting time of 5 s. Subsequently, the samples were centrifuged at a maximum speed for 3 min and the supernatants with the cell lysate were carefully extracted. To each lysate, 600 µL of 70% ethanol were added, mixed by pipetting, and transferred to the extraction columns of the RNeasy Mini Kit. The extraction of the RNA was performed according to the manufacturer instructions. The obtained RNA was stored at -80 ºC.

The complementary DNA (cDNA) was obtained using the qScript cDNA SuperMix kit (Quantabio, Beverly, MA, USA) and RT-PCR was performed as detailed in our previous study (Abad et al., 2022). This procedure was carried out to determine expression levels of IL-6, IL-10, IL-12 p35, IL-12 p40, TNF-α, and nucleotide-binding and oligomerization domain (NOD)-like receptors. The specific primers are detailed in Table 1.

The results obtained for the threshold cycles (Ct) were statistically analyzed by subtracting the mean of the Ct values corresponding to the housekeeping genes (GAPDH and HPRT1) from the Ct for amplification of studied genes ($\Delta Ct_{treatment} = Ct_{gene} - Ct_{housekeeping}$). The mean values of the negative controls were subtracted from the previously obtained value ($\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$). Finally, the relative gene expression was calculated and expressed as fold change ($2^{-\Delta\Delta Ct}$).

Statistical analysis

The analysis was performed using the statistical software GraphPad Prism v8.0.2 (GraphPad Software, San Diego, CA, USA). The normality of the data was checked with the Saphiro-Wilk test. To compare the means of three or more unpaired groups, an analysis of variance (ANOVA) was performed. Dunnet's post-hoc test was used as a multiple comparison test. Data that did not follow a normal distribution were submitted to the non-parametric Kruskal-Wallis test

followed by Dunn's test as a multiple comparison test. Differences with a p-value ≤ 0.05 were considered statistically significant.

Results and discussion

Histopathology

The results of the evaluation of PMN infiltration in ileum, cecum and colon are shown in Figure 2. The PMN counts in ileum and cecum (Figures 2A, 2B) were higher for the Clin group, showing significant differences respect to the control group ($p < 0.01$ and $p < 0.05$, respectively). However, the PMN counts in the colon of mice (Figure 2C) did not show significant differences between the control and the Clin group. The treatment with LF significantly decreased the elevated PMN counts caused by the antibiotic ($p < 0.01$ for nLF/Clin and $p < 0.05$ for sLF/Clin) only in the ileum (Figure 2A), reaching basal levels. For this reason, the ileum was the tissue chosen for the following experiments.

Therefore, inflammatory changes were limited to the presence of PMN cells in the ileum (Figure 3). In the control group (Figure 3A), the number of PMN cells was low; however, in the group of mice treated with clindamycin (Figure 3B), the presence of these cells was much more abundant, indicating an inflammatory process. In the groups of mice treated with both native and iron-saturated LF, the presence of PMN cells in the ileum was practically non-existent (Figures 3C, 3D). Finally, in the groups of mice treated with LF and Clin (Figures 3E, 3F), the presence of PMN cells in the ileum was very similar to that of the control group, and notably lower compared to the group treated only with the antibiotic.

Lipid and protein oxidation levels

Clindamycin did not modify MDA + 4-HDA levels (Figure 4A), but increased significantly the protein carbonyl levels in ileum up to almost 5-fold with respect to the control ($p < 0.0001$) (Figure 4B). The oral administration of nLF increased slightly the carbonyl levels in ileum respect to the control (Figure 4B) and sLF also modified the oxidative stress produced in lipids and proteins (Figure 4). However, sLF significantly reversed protein oxidative stress caused by clindamycin to basal levels when administered to antibiotic-treated mice ($p < 0.0001$).

In the study by Xiao et al. (2019), the authors analyzed the oxidative stress caused by different antibiotics *in vitro*, and concluded that clindamycin at 200 μg/mL caused oxidative lipid damage observed by the increase of MDA levels. These results do not coincide with ours, since clindamycin did not cause oxidative stress to lipids (Figure 4A). This difference in the results

could be mainly because their study was carried out *in vitro* in different cell lines, while our results derived from an *in vivo* experiment.

Milk proteins, such as LF, have antioxidant effects, by chelating metals and modulating enzymes involved in ROS production (Bielecka, Cichosz, & Czeczot, 2022). LF binds iron, which increases its bioavailability and decreases its pro-oxidant effects. LF is able to control ROS levels by uptaking free iron, which is involved in ROS production (Cutone et al., 2020). Therefore, it would be expected that nLF would have exerted greater antioxidant effect than sLF, since it is more available to capture iron from the medium. However, in our study, sLF has shown a greater effect than nLF, and this could be due to the fact that the iron-saturated protein is more stable and resistant to digestion (Elzoghby et al., 2020; Fan et al., 2022), which would allow it to reach the ileum and modulate the effect of clindamycin. Although sLF alone had a negative effect by generating lipid and protein oxidation, when it was administered with the antibiotic it had a positive effect, decreasing oxidation.

On the other hand, it could be possible that sLF loses part of its iron content as it passes through the gastrointestinal tract. It has been shown that the ferric ions are bound to LF by a strong bond that can resist pHs as low as 4 (Elzoghby et al., 2020), though acidic pHs, such as that of the stomach, can cause the release of iron from LF (Britton & Koldovsky, 1989). However, the degree of iron release from sLF when passing through the gastrointestinal tract can be very different according to species, although there are no available data about this.

Furthermore, previous studies have shown that both selenium-saturated LF (Burrow et al., 2011) and sLF (Burrow, Kanwar, & Kanwar, 2011) have antioxidant activity in human intestinal epithelial cells, interacting with redox systems and modifying the activity of antioxidant enzymes, being beneficial in helping to maintain the balance of oxidant/antioxidant systems.

Therefore, considering the findings of previous studies, it could be possible the existence of complementary antioxidant activity between LF molecules that have lost part of the iron and can take it up from the medium, and LF molecules still saturated that modulate the antioxidant enzymes.

In any case, further studies would be necessary to know the mechanism of action of LF and the effect that the level of iron saturation causes in its antioxidant activity. Although this mechanism of action is not known, it would be interesting to investigate whether the administration of sLF can be preventive when an antibiotic is going to be administered to reverse the negative effects of it.

Inflammatory cytokine expression levels

The results obtained in the present study show that clindamycin significantly increased the expression of some pro-inflammatory cytokines, such as IL-6 and TNF-α, by 1- and 2-fold change, respectively, compared with the control group (Figure 5).

IL-6 is a single-chain protein, originally identified as a B-cell differentiation factor. This cytokine is produced in T cells, B cells, monocytes and fibroblasts, and is involved in the maturation of antibody-producing cells. IL-6 can bind to its receptor IL-6R whether it is soluble or located on the cell surface. The expression of IL-6 is increased in inflammatory diseases, and a blockage of this cytokine allows the control of symptoms and a slowdown in the progression of the disease (Cronstein, 2007). Excessive secretion of IL-6 and its dysregulation may play an important role in the pathogenesis of many diseases, including IBD (Suzuki, Yoshinaga, & Tanabe, 2011). Thus, it has been reported in mice that inactivation and blockage of IL-6 and its receptor decrease the incidence of colitis, indicating a key contribution of IL-6 in this process (Sander et al., 2008). In our results, the pro-inflammatory effect of clindamycin was reversed by oral treatment of nLF and sLF, significantly decreasing the expression of IL-6 to control levels ($p < 0.01$ and $p < 0.05$, respectively, compared to the Clin group). However, nLF and sLF did not modify the expression levels of this cytokine by themselves (Figure 5A).

TNF-α is a key protein in the immune response, with multiple effects, from inflammation to apoptosis. TNF-α increases the production of other pro-inflammatory cytokines, such as IL-6, and promotes apoptosis by binding to its receptor (Victor & Gottlieb, 2002). It has been reported that some pro-inflammatory cytokines, such as TNF-α, decrease the expression of the tight junction structure and induce epithelial apoptosis (Suzuki, Yoshinaga, & Tanabe, 2011). In our study, there was a strong and statistically significant increase of TNF-α expression in the Clin group ($p < 0.001$ with respect to control); while the administration of nLF and sLF improved the situation of the ileum cells, significantly decreasing the effect of the antibiotic to basal levels ($p < 0.01$ and $p < 0.0001$ compared with Clin). Furthermore, the single treatment with LF did not modify TNF-α expression (Figure 5B).

In the study by Fan et al. (2022), the authors analyzed the protective effect of LF with different iron saturation in a murine model of a lipopolysaccharide (LPS)-induced intestinal inflammation. They concluded that LPS increased significantly the expression levels of IL-6 and TNF-α in the colon of mice. However, both apo- and holo-LF decreased significantly the levels of these inflammatory factors when administered orally to mice treated with LPS. These results are very similar to those obtained in the present study. Furthermore, it has been shown that doses of LF similar to those used in our study have effects on the intestinal immune system of mice (Wakabayashi et al., 2006; Bellés et al., 2022).

The main target of orally administrated LF is the intestinal immune system, since intact LF or its functional fragments, such as lactoferricin, are not transferred to the blood unless there is a lesion in the intestine (Wakabayashi et al., 2004b). It has been observed that, in inflammatory bowel diseases, IL-6 levels are elevated (Suzuki, Yoshinaga, & Tanabe, 2011). In our case, the increase in IL-6 experimented by the Clin group could indicate an activation of the intestinal immune system, which is reversed by treating the mice with LF (Figure 4A). This process of activation caused in the ileum by clindamycin could be seen by the increase in the expression of inflammatory cytokines and the production of ROS, although it was not an inflammation clearly observed macroscopically or under the microscope when the histological analysis of the tissue was performed (Figure 3).

Similar results have been obtained in previous studies with *in vitro* and *in vivo* models of LPS-induced inflammation, where LF modulated the immune response, downregulating pro-inflammatory cytokines, such as IL-6 and TNF-α, and enhancing the secretion of anti-inflammatory cytokines, like IL-10 and IL-4 (Legrand et al., 2005). Furthermore, oral administration of LF-derived lactoferricin decreased the inflammatory response in a murine model of intestinal dysfunction caused by *Escherichia coli*, suppressing the expression and release of IL-6 and TNF-α to basal levels (Zhang et al., 2019).

On the other hand, clindamycin treatment did not alter the expression levels of other inflammatory cytokines analyzed, like IL-10, IL-12 p35, IL-12 p40, and NOD-like receptors (Figure 6).

IL-10 is one of the most important anti-inflammatory cytokines, besides IL-35 and TGF-β (Sabat et al., 2010). Neither clindamycin nor LF, in any of its iron saturation states, showed any effect on the expression of IL-10 (Figure 6A). IL-10 is a homodimer synthesized by several type of cells. Monocytes and macrophages secrete IL-10 after endogenous or exogenous activation, and this interleukin decreases the production of inflammatory mediators (Sabat et al., 2010). In the study by Madsen et al. (2000), IL-10 knockout mice developed chronic enterocolitis and showed significantly increased levels of bacteria in the colonic mucosa, indicating that this cytokine plays an important role in controlling the intestinal microbiota and regulating the intestinal inflammation process.

The expression levels of the two subunits of interleukin-12, IL-12 p35 and IL-12 p40, were significantly increased by sLF treatment (Figures 6B, 6C). However, the expression of these subunits in the clindamycin group did not show significant differences compared to that of the control. This cytokine is a heterodimeric protein that has an important role as a mediator in the

host's defense against infections and cancer (Del Vecchio et al., 2007). Wakabayashi et al. (2006) analyzed the effect of orally administered bovine LF (8.2% of iron saturation) in the gene expression of the two subunits of interleukin-12 in the small intestine of mice. That study showed that IL-12 p35 and IL-12 p40 were upregulated by nLF administration, while in our study, sLF showed more effect than nLF.

In addition, Toll-like innate immune receptors (TLRs) and NOD-like receptors are also involved, with their signalling cascades, in the modulation of epithelial barrier repair and integrity (Parlato & Yeretssian, 2014). NOD-like receptors are strategically expressed in the intestine (Rubino et al., 2012). NOD1 and NOD2 play an important role in the activation of innate immune signalling after bacterial detection. These receptors distinguish between Gram-negative and Gram-positive bacteria by detecting specific peptidoglycan motifs. While NOD1 recognizes D-glutamyl-meso-diaminopimelic acid found in Gram-negative and certain Gram-positive bacteria, NOD2 detects the muramyl-dipeptide common in Gram-positive bacteria (Parlato & Yeretssian, 2014). NOD1 is highly expressed in intestinal epithelial cells. This receptor has not been directly associated with intestinal inflammation, but instead it has been given a role in regulating host responses to normal intestinal microbiota and enteric pathogens in intestinal cells (Rubino et al., 2012). On the other hand, elevated levels of NOD2 have been observed in patients with IBD. Indeed, there is evidence that inflammation during IBD drives increased expression of NOD2 (Parlato & Yeretssian, 2014). However, the results obtained in our study did not show any significant modification in the expression of NOD1 and NOD2 (Figures 6D, 6E).

This study was part of a more extensive *in vivo* experiment, in which parameters such as composition of gut microbiota and TLR receptor expression were analyzed. Those analyses showed that clindamycin induces certain alterations in the expression of TLR receptors and in the composition of the intestinal microbiota, reducing the presence of bacteria with anti-inflammatory properties. However, the administration of LF, both native and iron-saturated, to mice treated with clindamycin managed to re-establish the levels of the anti-inflammatory bacteria. In addition, sLF also restored the levels of TLR expression altered by clindamycin (Bellés et al., 2022).

Therefore, all the results obtained in this *in vivo* experiment could reinforce the fact that LF can be considered as an interesting ingredient to be used in functional foods, since it has been shown to contribute to the maintenance of intestinal health. In general, sLF has shown better effects against clindamycin than nLF, although further studies should be carried out to find out which is the role of iron in the protective activity of LF against the effect of this antibiotic.

Conclusions

Although clindamycin did not cause clear inflammation of the small intestine in mice, it did activate an inflammatory process, recruiting polymorphonuclear cells in the ileum. This inflammation process seemed to improve with the ingestion of lactoferrin, both native and iron-saturated, restoring the presence of these defensive cells in the ileum to basal levels. The results of this study, supported by previous studies, confirmed that oral administration of lactoferrin protects against the negative effects of clindamycin in oxidation extent and expression of inflammatory mediators in the ileum of mice. Lactoferrin reversed the increase in the protein carbonyl content and reduced the expression of pro-inflammatory cytokines, such as IL-6 and TNF-α, altered by the action of clindamycin. Although these studies should be expanded and continued to better understand the mechanism of action of lactoferrin in the intestine, given the large number of beneficial properties for health that this protein presents, it could be an interesting ingredient for functional foods, being very useful as “protective” when taking antibiotics such as clindamycin.

Acknowledgements

This work was funded by grants from the Spanish Ministry of Economy, Industry and Competitiveness and the European Regional Development Fund (ERDF/FEDER) (AGL2017-82987), the European Social Found (ESF) and the Aragon Regional Government (A20_23R).

Competing interests

The authors declare there are no competing interests.

Data availability

Data generated or analyzed during this study are available from the corresponding author upon reasonable request.

References

Abad, I., Sangüesa, A., Ubieto, M., Carramiñana, J. J., Pérez, M. D., Buey, B., Mesonero, J. E., Grasa, L., & Sánchez, L. (2022). Protective effect of bovine lactoferrin against *Cronobacter sakazakii* in human intestinal Caco-2/TC7 cells. *International Dairy Journal*, 133, 105428. https://doi.org/10.1016/j.idairyj.2022.105428

Adlerova, L., Bartoskova, A., & Faldyna, M. J. V. M. (2008). Lactoferrin: a review. *Veterinarni Medicina*, 53(9), 457-468. https://doi.org/10.17221/1978-VETMED

Bellés, A., Aguirre-Ramírez, D., Abad, I., Parras-Moltó, M., Sánchez, L., & Grasa, L. (2022). Lactoferrin modulates gut microbiota and Toll-like receptors (TLRs) in mice with dysbiosis induced by antibiotics. *Food & Function*, 13(10), 5854-5869.

https://doi.org/10.1039/D2FO00287F

Bielecka, M., Cichosz, G., & Czeczot, H. (2022). Antioxidant, antimicrobial and anticarcinogenic activities of bovine milk proteins and their hydrolysates-A review. *International Dairy Journal*, 127, 105208. https://doi.org/10.1016/j.idairyj.2021.105208

Britton, J. R., & Koldovsky, O. (1989). Gastric luminal digestion of lactoferrin and transferrin by preterm infants. *Early Human Development*, 19(2), 127-135. https://doi.org/10.1016/0378-3782(89)90123-0

Buey, B., Bellés, A., Latorre, E., Abad, I., Pérez, M. D., Grasa, L., Mesonero, J. E., & Sánchez, L. (2021). Comparative effect of bovine buttermilk, whey, and lactoferrin on the innate immunity receptors and oxidative status of intestinal epithelial cells. *Biochemistry and Cell Biology*, 99(1), 54-60. https://doi.org/10.1139/bcb-2020-0121

Buffie, C. G., Jarchum, I., Equinda, M., Lipuma, L., Gobourne, A., Viale, A., Ubeda, C., Xavier, J., & Pamer, E. G. (2012). Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to *Clostridium difficile*-induced colitis. *Infection and Immunity*, 80(1), 62-73. https://doi.org/10.1128/iai.05496-11

Burrow, H., Kanwar, R. K., & Kanwar, J. K. (2011). Antioxidant enzyme activities of iron-saturated bovine lactoferrin (Fe-bLf) in human gut epithelial cells under oxidative stress. *Medicinal Chemistry*, 7(3), 224-230. https://doi.org/10.2174/157340611795564286

Burrow, H., Kanwar, R. K., Mahidhara, G., & Kanwar, J. R. (2011). Effect of selenium-saturated bovine lactoferrin (Se-bLF) on antioxidant enzyme activities in human gut epithelial cells under oxidative stress. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, 11(8), 762-771. https://doi.org/10.2174/187152011797378616

Chelombitko, M. A. (2018). Role of reactive oxygen species in inflammation: a minireview. *Moscow University Biological Sciences Bulletin*, 73, 199-202. https://doi.org/10.3103/S009639251804003X

Conesa, C., Bellés, A., Grasa, L., & Sánchez, L. (2023). The role of lactoferrin in intestinal health. *Pharmaceutics*, 15(6), 1569. https://doi.org/10.3390/pharmaceutics15061569

Cronstein, B. N. (2007). Interleukin-6. *Bulletin of the NYU Hospital for Joint Diseases*, 65(1), 11-15.

Cutone, A., Rosa, L., Ianiro, G., Lepanto, M. S., Bonaccorsi di Patti, M. C., Valenti, P., & Musci, G. (2020). Lactoferrin's anti-cancer properties: Safety, selectivity, and wide range of action. *Biomolecules*, 10(3), 456. https://doi.org/10.3390/biom10030456

Del Vecchio, M., Bajetta, E., Canova, S., Lotze, M. T., Wesa, A., Parmiani, G., & Anichini, A. (2007). Interleukin-12: biological properties and clinical application. *Clinical Cancer Research*, 13(16), 4677-4685. https://doi.org/10.1158/1078-0432.CCR-07-0776

Elzoghby, A. O., Abdelmoneem, M. A., Hassanin, I. A., Abd Elwakil, M. M., Elnaggar, M. A., Mokhtar, S., Fang, J. Y., & Elkhodairy, K. A. (2020). Lactoferrin, a multi-functional glycoprotein: Active therapeutic, drug nanocarrier & targeting ligand. *Biomaterials*, 263, 120355. https://doi.org/10.1016/j.biomaterials.2020.120355

Fan, L. L., Yao, Q. Q., Wu, H. M., Wen, F., Wang, J. Q., Li, H. Y., & Zheng, N. (2022). Protective effects of recombinant lactoferrin with different iron saturations on enteritis injury in young mice. *Journal of Dairy Science*, 105(6), 4791-4803. https://doi.org/10.3168/jds.2021-21428

Gonzalo, S., Grasa, L., Arruebo, M. P., Plaza, M. Á., & Murillo, M. D. (2011). Extracellular signal-regulated kinase (ERK) is involved in LPS-induced disturbances in intestinal motility. *Neurogastroenterology & Motility*, 23(2), 80-90. https://doi.org/10.1111/j.1365-2982.2010.01632.x

Graikini, D., Conesa, C., Abad, I., Pérez, M. D., & Sánchez, L. (2023). Evaluation of *in vitro* antirotaviral activity of lactoferrin from different species using a human intestinal model. *International Dairy Journal*, 105818. https://doi.org/10.1016/j.idairyj.2023.105818

Guillouzo, A., & Guguen-Guillouzo, C. (2020). Antibiotics-induced oxidative stress. *Current Opinion in Toxicology*, 20, 23-28. https://doi.org/10.1016/j.cotox.2020.03.004

Korcheva, V., Wong, J., Corless, C., Iordanov, M., & Magun, B. (2005). Administration of ricin induces a severe inflammatory response via nonredundant stimulation of ERK, JNK, and P38 MAPK and provides a mouse model of hemolytic uremic syndrome. *The American Journal of Pathology*, 166(1), 323-339. https://doi.org/10.1016/S0002-9440(10)62256-0

Legrand, D., Elass, E., Carpentier, M., & Mazurier, J. (2005). Lactoferrin: Lactoferrin: a modulator of immune and inflammatory responses. *Cellular and Molecular Life Sciences*, 62, 2549-2559. https://doi.org/10.1007/s00018-005-5370-2

Li, L., Ren, F., Yun, Z., An, Y., Wang, C., & Yan, X. (2013). Determination of the effects of lactoferrin in a preclinical mouse model of experimental colitis. *Molecular Medicine Reports*, 8(4), 1125-1129. https://doi.org/10.3892/mmr.2013.1632

Madsen, K. L., Doyle, J. S., Tavernini, M. M., Jewell, L. D., Rennie, R. P., & Fedorak, R. N. (2000). Antibiotic therapy attenuates colitis in interleukin 10 gene-deficient mice. *Gastroenterology*, 118(6), 1094-1105. https://doi.org/10.1016/S0016-5085(00)70362-3

Nair, A. B., & Jacob, S. (2016). A simple practice guide for dose conversion between animals and human. *Journal of Basic and Clinical Pharmacy*, 7(2), 27. https://doi.org/10.4103/0976-0105.177703

Orsi, N. (2004). The antimicrobial activity of lactoferrin: current status and perspectives. *Biometals*, 17, 189-196. https://doi.org/10.1023/B:BIOM.0000027691.86757.e2

Parlato, M., & Yeretssian, G. (2014). NOD-like receptors in intestinal homeostasis and epithelial tissue repair. *International Journal of Molecular Sciences*, 15(6), 9594-9627. https://doi.org/10.3390/ijms15069594

Robertson, S. J., Zhou, J. Y., Geddes, K., Rubino, S. J., Cho, J. H., Girardin, S. E., & Philpott, D. J. (2013). Nod1 and Nod2 signaling does not alter the composition of intestinal bacterial communities at homeostasis. *Gut Microbes*, 4(3), 222-231. https://doi.org/10.4161/gmic.24373

Rubino, S. J., Selvanantham, T., Girardin, S. E., & Philpott, D. J. (2012). Nod-like receptors in the control of intestinal inflammation. *Current Opinion in Immunology*, 24(4), 398-404. https://doi.org/10.1016/j.coi.2012.04.010

Sabat, R., Grütz, G., Warszawska, K., Kirsch, S., Witte, E., Wolk, K., & Geginat, J. (2010). Biology of interleukin-10. *Cytokine & Growth Factor Reviews*, 21(5), 331-344. https://doi.org/10.1016/j.cytogfr.2010.09.002

Sander, L. E., Obermeier, F., Dierssen, U., Kroy, D. C., Singh, A. K., Seidler, U., Streetz, K. L., Lutz, H. H., Müller, W., Tacke, F., & Trautwein, C. (2008). Gp130 signaling promotes development of

acute experimental colitis by facilitating early neutrophil/macrophage recruitment and activation. *The Journal of Immunology*, 181(5), 3586-3594. https://doi.org/10.4049/jimmunol.181.5.3586

Schenk, M., & Mueller, C. (2008). The mucosal immune system at the gastrointestinal barrier. *Best Practice & Research Clinical Gastroenterology*, 22(3), 391-409. https://doi.org/10.1016/j.bpg.2007.11.002

Shekhar, S., & Petersen, F. C. (2020). The dark side of antibiotics: adverse effects on the infant immune defense against infection. *Frontiers in Pediatrics*, 8, 544460. https://doi.org/10.3389/fped.2020.544460

Suzuki, T., Yoshinaga, N., & Tanabe, S. (2011). Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. *Journal of Biological Chemistry*, 286(36), 31263-31271. https://doi.org/10.1074/jbc.M111.238147

Victor, F. C., & Gottlieb, A. B. (2002). TNF-alpha and apoptosis: implications for the pathogenesis and treatment of psoriasis. *Journal of Drugs in Dermatology*, 1(3), 264-275. PMID: 12851985

Wakabayashi, H., Kurokawa, M., Shin, K., Teraguchi, S., Tamura, Y., & Shiraki, K. (2004a). Oral lactoferrin prevents body weight loss and increases cytokine responses during herpes simplex virus type 1 infection of mice. *Bioscience, Biotechnology, and Biochemistry*, 68(3), 537-544. https://doi.org/10.1271/bbb.68.537

Wakabayashi, H., Kuwata, H., Yamauchi, K., Teraguchi, S., & Tamura, Y. (2004b). No detectable transfer of dietary lactoferrin or its functional fragments to portal blood in healthy adult rats. *Bioscience, Biotechnology, and Biochemistry*, 68(4), 853-860. https://doi.org/10.1271/bbb.68.853

Wakabayashi, H., Takakura, N., Yamauchi, K., & Tamura, Y. (2006). Modulation of immunity-related gene expression in small intestines of mice by oral administration of lactoferrin. *Clinical and Vaccine Immunology*, 13(2), 239-245. https://doi.org/10.1128/CVI.13.2.239-245.2006

Wang, B., Timilsena, Y. P., Blanch, E., & Adhikari, B. (2019). Lactoferrin: Structure, function, denaturation and digestion. *Critical Reviews in Food Science and Nutrition*, 59(4), 580-596. https://doi.org/10.1080/10408398.2017.1381583

Welter-Stahl, L., Ojcius, D. M., Viala, J., Girardin, S., Liu, W., Delarbre, C., Philpott, D., Kelly, K. A., & Darville, T. (2006). Stimulation of the cytosolic receptor for peptidoglycan, Nod1, by infection with *Chlamydia trachomatis* or *Chlamydia muridarum*. *Cellular Microbiology*, 8(6), 1047-1057. https://doi.org/10.1111/j.1462-5822.2006.00686.x

Xiao, Y., Xiong, T., Meng, X., Yu, D., Xiao, Z., & Song, L. (2019). Different influences on mitochondrial function, oxidative stress and cytotoxicity of antibiotics on primary human neuron and cell lines. *Journal of Biochemical and Molecular Toxicology*, 33(4), e22277. https://doi.org/10.1002/jbt.22277

Zhang HaiWen, Z. H., Hua Rui, H. R., Zhang BingXi, Z. B., Guan QingFeng, G. Q., Zeng JiFeng, Z. J., Wang XueMei, W. X., & Wang BeiBei, W. B. (2019). Oral administration of bovine lactoferrin-derived lactoferricin (Lfcin) B could attenuate enterohemorrhagic Escherichia coli O157:H7 induced intestinal disease through improving intestinal barrier function and microbiota. Journal of Agricultural and Food Chemistry, 67(14), 3932-3945. https://doi.org/10.1021/acs.jafc.9b00861

Table 1. Primer sequences used for RT-PCR analysis of housekeeping genes, interleukins, and NOD-like receptors. Fw: Forward primer, Rv: Reverse primer.

Gene	Primer (5' - 3')		Reference
GAPDH	Fw	CATGACCACAGTCCATGCCATCACT	Buey et al., 2021
	Rv	TGAGGTCCACCACCCTGTTGCTGTA	
HPRT1	Fw	CTGACCTGCTGGATTACA	Buey et al., 2021
	Rv	GCGACCTTGACCATCTTT	
IL-6	Fw	TCCTACCCCAATTTCCAATGC	Welter-Stahl et al., 2006
	Rv	TGAATTGGATGGTCTTGGTCCT	
IL-10	Fw	GGACAACATACTGCTAACCGAC	Wakabayashi et al., 2006
	Rv	AAAATCACTCTTCACCTGCTCC	
IL-12 p35	Fw	CATCGATGAGCTGATGCAGT	Wakabayashi et al., 2006
	Rv	CAGATAGCCCATCACCCTGT	
IL-12 p40	Fw	TGGAAGCACGGCAGCAGAATAAAT	Wakabayashi et al., 2006
	Rv	TGCGCTGGATTCGAACAAAGAACT	
TNF-α	Fw	AAATGGGCTTTCCGAATTCA	Korcheva et al., 2005
	Rv	CAGGGAAGAATCTGGAAAGGT	
NOD1	Fw	TCCCTTGCCTGTGAGCAGAAAGTA	Robertson et al., 2013
	Rv	GTGGGTATGTGCCATGCTTTGCTT	
NOD2	Fw	GGAGGAGCTTCCAGGAGTTT	Wakabayashi et al., 2006
	Rv	ACTCGTCCAAGCCATCAAAG	

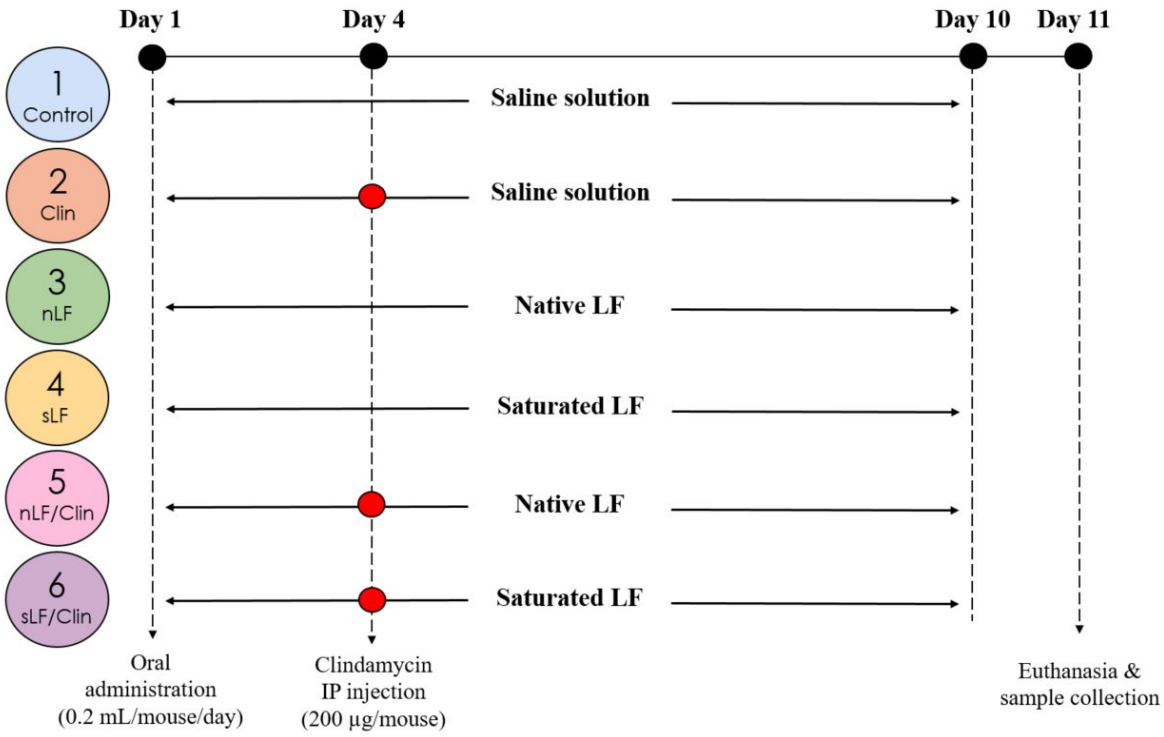

Figure 1. Division of the six groups of mice, 5 males per group. Control: oral administration of saline, Clin: oral administration of saline and intraperitoneal injection of clindamycin, nLF: oral administration of native lactoferrin, sLF: oral administration of iron-saturated lactoferrin, nLF/Clin: oral administration of native lactoferrin and intraperitoneal injection of clindamycin, sLF/Clin: oral administration of iron-saturated lactoferrin and intraperitoneal injection of clindamycin. During the first 10 days, all animals received an oral administration of saline for groups 1 and 2, native LF for groups 3 and 5, or saturated LF for groups 4 and 6. Additionally, groups treated with Clin (2, 5 and 6) received, on day 4, a single intraperitoneal injection of clindamycin. On day 11 of the experiment, the mice were humanely euthanized and organs were collected.

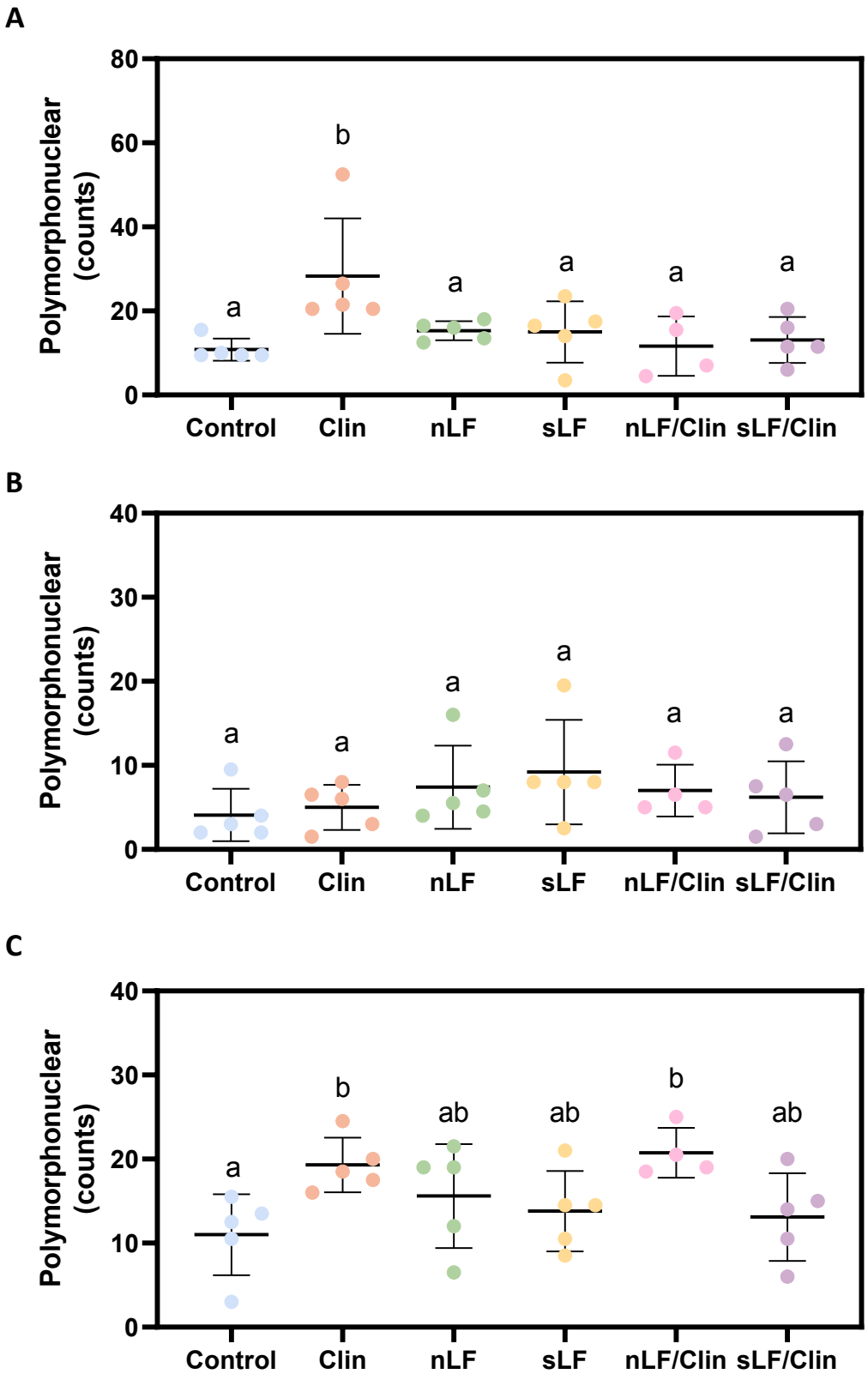

Figure 2. Polymorphonuclear counts in the (A) ileum, (B) cecum and (C) colon of the different groups of mice. Clin: clindamycin; nLF: native LF; sLF: iron-saturated LF. The horizontal line in the middle represents the mean (n = 5). Counts of different groups without a common letter differ statistically, $p < 0.05$.

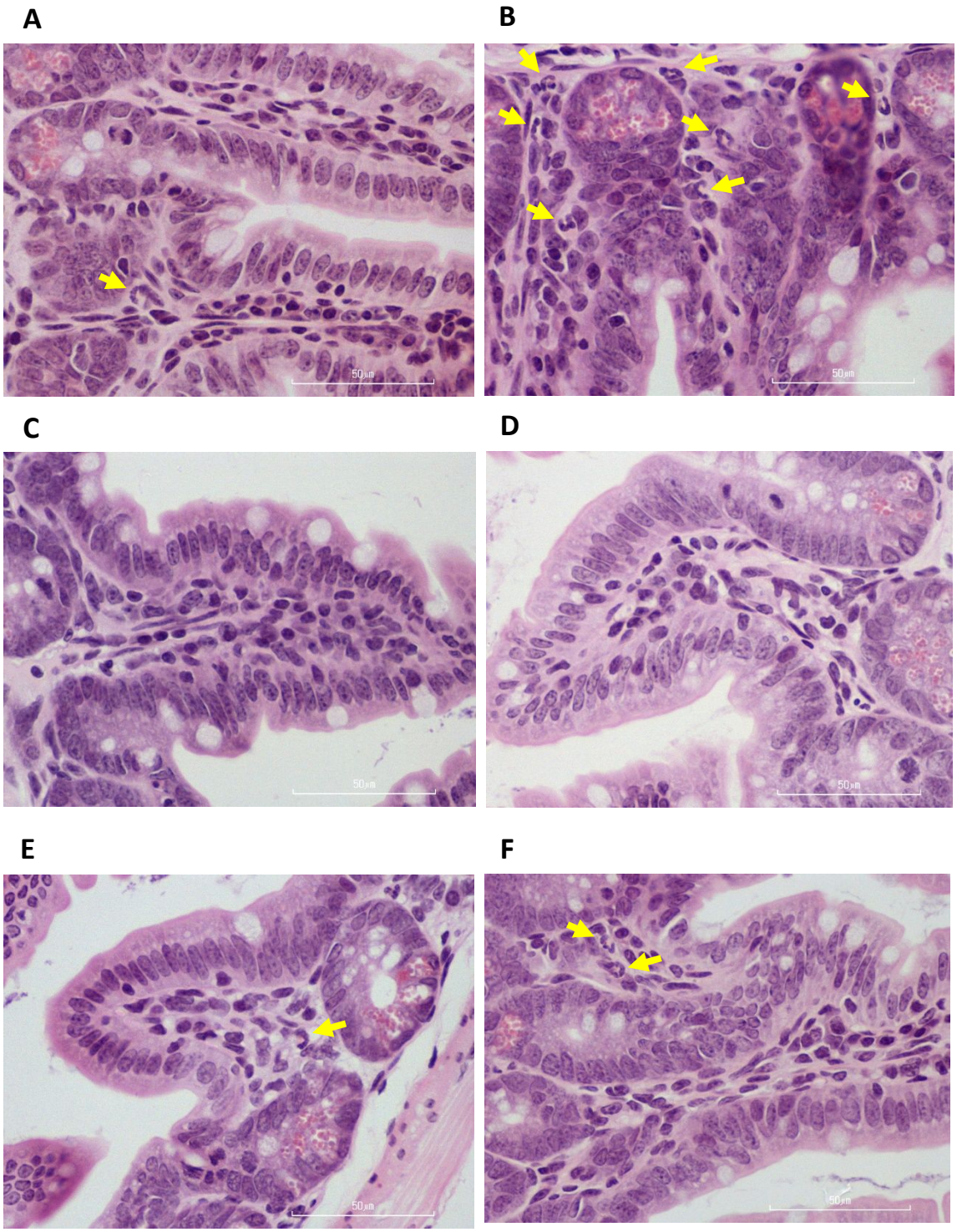

Figure 3. Polymorphonuclear cells in the ileum of mice of different groups. (A) Control group, (B) Clin group, (C) nLF group, (D) sLF group, (E) nLF/Clin group, (F) sLF/Clin group. Yellow arrows indicate the PMN cells. Staining with hematoxylin and eosin.

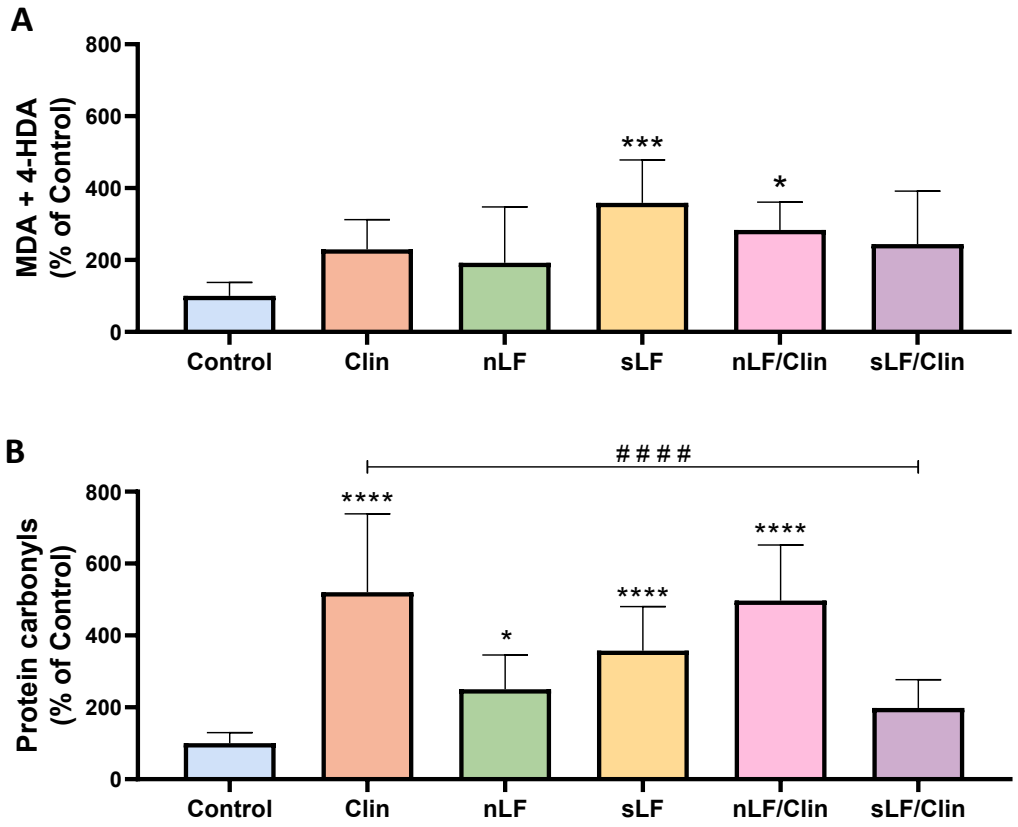

Figure 4. Effect of bovine LF on oxidative stress caused by clindamycin in mice ileum. (A) Levels of lipid peroxidation, determined by the concentration of MDA + 4-HDA. (B) Protein oxidation, determined by the measurement of carbonyls level. Clin: clindamycin; nLF: native LF; sLF: iron-saturated LF. The values represent the mean ± standard deviation of two replicates in five mice (n = 10). Asterisk indicates significant differences respect to Control (*p< 0.05, ***p< 0.001, ****p< 0.0001). Hash indicates significant differences respect to Clin (#### p< 0.0001).

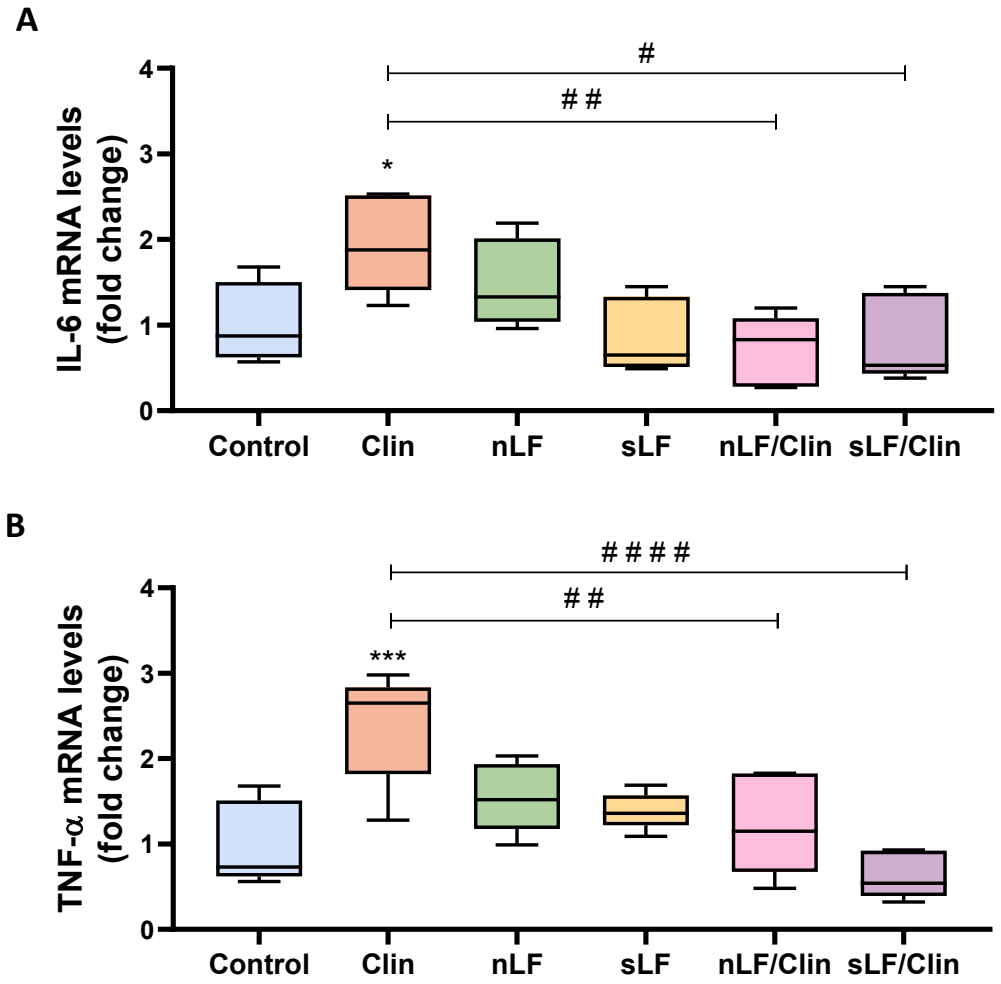

Figure 5. Effect of bovine LF on the expression levels of (A) IL-6 and (B) TNF-α in mouse ileum. Clin: clindamycin; nLF: native LF; sLF: iron-saturated LF. The horizontal line in the middle of each box represents the median, while the top and bottom borders of the boxes represent the 75 and 25 percentiles, respectively, n = 5. Asterisk indicates significant differences respect to Control (*p< 0.05, ***p< 0.001). Hash indicates significant differences respect to Clin ($^{\#}$p< 0.05, $^{\#\#}$p< 0.01, $^{\#\#\#\#}$p<0.0001).

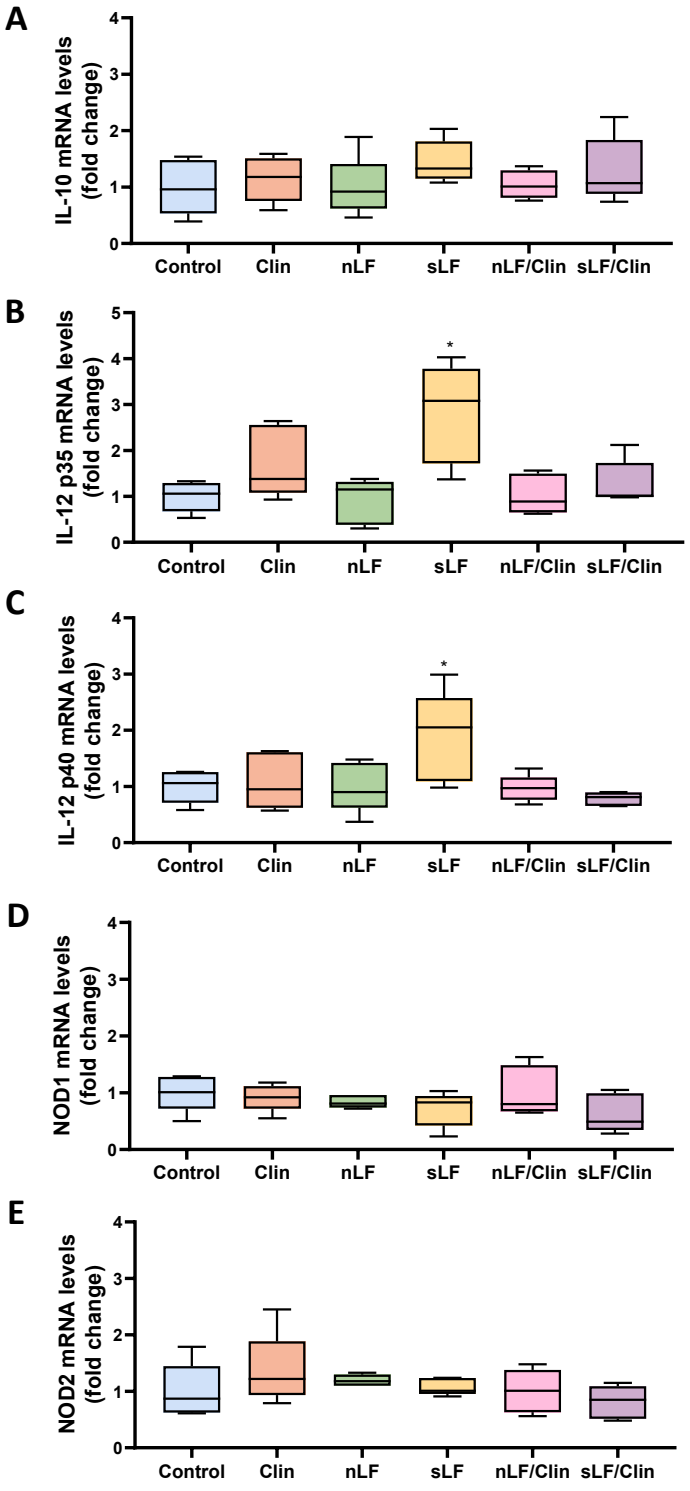

Figure 6. Effect of bovine LF on the expression of (A) IL-10, (B) IL-12 p35, (C) IL-12 p40, (D) NOD1 and (E) NOD2 in mice ileum. Clin: clindamycin; nLF: native LF; sLF: iron-saturated LF. The horizontal line in the middle of each box represents the median, while the top and bottom borders of the boxes represent the 75 and 25 percentiles, respectively, n = 5. Asterisk indicates significant differences respect to Control (*p< 0.05).