Buttermilk and Whey as functional foods to ameliorate clindamycin-induced changes in mouse intestine: modulation of intestinal motility and Toll-like receptors expression

Running title: Buttermilk and Whey Modulate Motility and TLRs

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ABSTRACT

Antibiotic treatment is one of the main causes of intestinal dysbiosis, leading, in turn, to other intestinal alterations given the multiple relationships of the microbiota with gut health. Whey and buttermilk are two by-products from the dairy industry with numerous bioactive components. This study aimed to assess the potential of two formulas, containing a mixture of lactoferrin, milk fat globule membrane (MFGM), and whey or buttermilk, to reverse the negative effects of clindamycin on gut motility, Toll-like receptors (TLRs) expression, and oxidative stress in the intestine. For this purpose, a murine model of intestinal dysbiosis was established by clindamycin treatment. Male C57BL/6 mice were treated with saline (Control), clindamycin (Clin), a formula containing whey (F1), or buttermilk (F2) supplemented with lactoferrin and MFGM, Clin+F1 or Clin+F2. Clin delayed the whole gut transit, reduced the response to acetylcholine, decreased TLR2 expression, and increased TLR4 expression in the intestine. F1 and F2 formulas reversed the effects of Clin, restoring TLR2 receptor levels and normalizing intestinal dysmotility. These results indicate that whey- and buttermilk-based formulas supplemented with lactoferrin and MFGM could be used as functional foods to prevent or treat motility disorders and restore some components of the immune system after antibiotic treatment.

INTRODUCTION

Intestinal microbiota and its metabolites are essential for the normal development of both the immune system and intestinal motility. In fact, it has been shown that germ-free animals display abnormal immune responses [1], delayed intestinal transit, and decreased intestinal contractility [2]. Therefore, gut microbiota plays a crucial role in preserving gut homeostasis.

The use of broad-spectrum antibiotics has some drawbacks, such as the spread of resistance, and changes in the composition and function of the microbiota (dysbiosis), which in turn alters immune responses [3]. Furthermore, gut dysbiosis affects the structural and functional integrity of the enteric nervous system (ENS), resulting in intestinal motility dysfunction [4]. Another important side effect of bactericidal antibiotics is the stimulation of the production of reactive oxygen species (ROS) [5]. A basic level of ROS is essential for certain biological processes. However, an excess of these molecules in the organism causes oxidative damage to DNA, proteins, and membrane lipids, collectively known as oxidative stress [6].

Toll-like receptors (TLRs) are an important family of pattern recognition receptors (PRRs) expressed in immune cells, but also in enteric neurons [7] and intestinal epithelial cells [8]. These receptors are key agents of the innate immune system, as they recognize microbe or pathogen-associated molecular patterns (MAMPs or PAMPs, respectively) and activate intracellular signalling cascades, that result in the release of cytokines, chemokines, and interferon, thus contributing to host defence responses. These receptors recognize a wide variety of microorganisms, however, in this work, we focus on TLRs specialised in the recognition of bacterial ligands: TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9 [8]. In addition, the expression of some TLRs in the ENS suggests that microbiota may regulate gastrointestinal motility through these immune receptors [9-11]. Changes in TLRs have been detected in *in vitro* and mouse models treated with antibiotics [12,13]. The overexpression or inhibition of these receptors could disrupt gut homeostasis leading to different pathological conditions [14].

The relationship between diet and the prevention/reduction of certain diseases has been confirmed by several scientific studies. These findings have led to a change in the demands of consumers, who, in addition to being concerned about the nutritional value of foods, want to consume functional ingredients to prevent nutrition-related diseases and improve their physical and mental well-being [15,16]. There is currently a growing interest in identifying bioactive ingredients that provide physiological benefits beyond simple nutritional functions. Milk is an excellent source of bioactive compounds and, as a result of the high demand for novel, high-value-added, and affordable functional ingredients, dairy by-products are getting more attention. Whey and buttermilk

are two important by-products of the dairy industry with outstanding functional and nutritional properties. Whey is obtained after casein coagulation during cheese manufacture, and it is an important source of lactose and bioactive proteins. The major components of whey proteins include β-lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulins, bovine serum albumin (BSA), lactoferrin and lactoperoxidase. Buttermilk is the aqueous part obtained during butter production, containing lactose, casein, whey proteins, and milk fat globule membrane (MFGM) compounds. This biological membrane is mainly composed of a complex mixture of proteins, polar (phospholipids and sphingolipids) and apolar (triglycerides) lipids [17]. Whey proteins, as well as proteins and polar lipids present in MFGM, have received special attention since they possess many biological properties, such as anticancer, antihypertensive, antimicrobial, immunomodulatory, and antioxidant activity [17-19].

According to current knowledge, whey and buttermilk appear to be a rich source of bioactive compounds that could be used in the functional food and nutraceutical market, thus valorising these by-products, and avoiding the environmental problems related to their disposal. Therefore, this study aimed to investigate the ability of whey- and buttermilk-based formulas, supplemented with lactoferrin and MFGM, to modulate the effects induced by clindamycin on intestinal motor function, TLR expression, and production of ROS in mouse intestine.

MATERIALS AND METHODS

Preparation of buttermilk and whey-based formulas

Raw bovine milk was processed as detailed previously [20] to obtain whey and buttermilk. Both formulas were enriched with native bovine lactoferrin (nLF) donated by Tatua Nutritionals (Morrinsville, New Zealand) and used as previously described [12]. Formula 1 (F1) was composed of a whey base, supplemented with nLF and MFGM. To prepare it, freeze-dried whey was rehydrated, considering its dry matter (0.068 g/ml). Once dissolved in 50 ml of miliQ water, nLF was added at a concentration of 175 mg/ml. This process was carried out under constant agitation at 4 °C to ensure its correct dissolution. Finally, MFGM precipitate obtained by centrifugation of 50 ml of buttermilk was added. Formula 2 (F2) was prepared with a base of 50 ml of buttermilk, to which nLF and MFGM were added at the same concentrations as in F1.

Animal housing and treatments

All procedures were carried out in accordance with Project Licence PI40/17 and were authorized by the Ethics Committee for Animal Experiments of the University of Zaragoza (Spain). Animal care and use was carried out in accordance with the Spanish

Policy for Animal Protection RD53/2013, which complies with the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. 30 male C57BL/6 mice (8–12 weeks old, Janvier Labs, Le Genest-Saint-Isle, France) were randomly divided into 6 groups (n = 5 per group): Control, clindamycin (Clin), F1, F2, Clin+F1 and Clin+F2. Control group received saline solution orally by gastric gavage for 10 days. Clin group was gavaged for 10 days with saline solution, and on day 4 received a single IP injection of 200 µg of clindamycin (Normon Laboratories, Spain) diluted in 200 µl of saline. Mice from the groups F1 and F2 were treated for 10 days orally by gastric gavage with 200 µl F1, or F2, respectively. Mice from Clin+F1 and Clin+F2 groups were gavaged for 10 days with 200 µl F1 or F2, and on day 4 received an IP injection of 200 µg of clindamycin.

Mice were kept in a conventional laboratory animal facility at the University of Zaragoza at temperatures between 20 and 22 °C, under a 12 h light/dark cycle, with free access to chow and water.

Determination of gastrointestinal (GI) transit

At the end of the treatments, mice were transferred to individual cages with very little bedding and left to acclimatize to the cage for 1 hour. Mice were gavaged with 200 µl of an Evans blue marker (5 % Evans blue, 5 % gum arabic). Once the drug was given to the animals, mice were returned to their individual cages. All cages were constantly monitored until the excretion of the first Evans blue-stained faecal pellet. The whole GI transit time was measured by recording the duration it took for the mice to excrete blue-stained faeces.

Muscle contractility studies

Mice were euthanized by cervical dislocation and tissue samples of distal ileum were collected and placed immediately in ice-cold Krebs buffer. Ileum segments (15 mm length) were mounted in the longitudinal direction of the smooth muscle fibres in an organ bath, containing Krebs solution kept at 37 °C and continuously gassed with 95 % O_2 and 5 % CO_2. An isometric force transducer (Pioden UF1, Graham Bell House, Canterbury, UK) was connected to each segment and it was passively stretched to an initial tension of 0.5 g. The mechanical activity signal was amplified using the Mac Lab Bridge Amp (AD Instruments Inc., Milford, MA, USA) with a range of 2 mV, and recorded using the Mac Lab System/8e computer program (AD Instruments Inc., Milford, MA, USA) for later analysis. After 1 hour equilibration period in Krebs solution, each intestinal segment was incubated for 3 minutes with acetylcholine (ACh, 100 µM). ACh-induced contractions were measured as integrated mechanical activity (IMA) as follows: IMA = $A_1 – A_0$, where

A_1 and A_0 are the integrated area per second after and before adding ACh, respectively. The IMA was expressed as milliNewtons per second. The ACh contractions of the different groups of animals were expressed as a percentage of Control group (100%).

RNA Extraction and RT-PCR

Colon samples were collected and preserved in RNAlater solution (Ambion, Thermo Fisher Scientific, Madrid, Spain) for 24 hours and then stored at -80 °C. Total RNA was isolated from colon samples using the NZY Total RNA Isolation kit (NZYTech, Lisbon, Portugal). The extracted RNA (1 µg) was used as a template for first-strand cDNA synthesis using qScript cDNA SuperMix kit (Quantabio, Beverly, MA, USA). cDNAs obtained by reverse transcription were used to determine mRNA expression levels of TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9. Real-time PCR was carried out on the StepOnePlus Real-Time PCR System (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). The specific primers used are detailed in Table 1, as well as the housekeeping primers (GAPDH and HPRT). Each sample was run in triplicate and the mean C_T was determined from the three runs. Relative expression levels of genes were calculated using the $2^{-\Delta\Delta CT}$ method, explained in detail in previous studies [12].

Assessment of oxidative stress

Pieces of colon were collected, washed with 0.9% NaCl and stored at -80 °C until analysis. Tissue samples were homogenized in cold Tris buffer (50 mM, pH 7.4) using Yellowline DI 25 Ultra Turrax (IKA-Werke, Staufen, Germany). After centrifugation (10 min at 4°C and 3000g), the homogenate 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 [21]. Briefly, MDA + 4-HDA reacts with N-methyl-2-phenylindole, yielding a stable chromophore, which can be measured spectrophotometrically at an absorbance of 586 nm. 1,1,3,3-tetramethoxypropane was used as the standard. Results were expressed as nmol MDA + 4-HDA mg-1 of protein. Protein oxidation was analysed by measuring protein carbonyl content as previously described[21]. Tissue homogenates were incubated with 2,4-dinitrophenylhydrazine (DNPH) at 37 °C for 1 hour. Finally, protein carbonyl concentration was measured spectrophotometrically at 375 nm using guanidine as a blank. The results were expressed as nmol carbonyl groups mg-1 of protein.

Data analysis and statistics

Differences of the different groups of treatment with respect to the Control or Clin group were evaluated using an unpaired T-test or Mann-Whitney U test as appropriate. The data were analysed using the software GraphPad Prism version 8.00 and the differences between groups with p values <0.05 were considered statistically significant.

RESULTS

Effects on intestinal motility

The whole intestinal transit (Fig. 1) and muscle contractility studies (Fig. 2) were performed to evaluate alterations in intestinal motility in the different groups of mice. Clindamycin-treated mice exhibited delayed excretion of blue-pigmented pellets compared to Control mice (377±39.85 vs. 261±30.36 min; $p<0.05$). This finding indicates that antibiotic treatment significantly delays gastrointestinal transit in mice. Neither the F1 (273.2±58.48 min) nor the F2 milk formula (223.6± 42.15 min) significantly modified intestinal transit compared to the Control group. When F1 and F2 were administered to clindamycin-treated mice the antibiotic-induced delay in intestinal transit was not observed (242.8±49.16 min in Clin+F1; 309.8±21.89 min in Clin+F2), and significant differences were observed when comparing the intestinal transit of Clin+F1 and Clin+F2 groups with the Clin group (Fig. 1).

Fig.2 shows the variations in the motor response to ACh in the longitudinal muscle of the ileum among mice from the different treatment groups. ACh induced contraction in the ileum of all mice. In Clin group, the contractile response induced by ACh was smaller compared to Control mice. F1 and F2 treatments did not produce any significant change in the response to ACh compared to either the Control or Clin groups. In Clin-treated mice, administration of F1 or F2 partially reverted the reduced response to ACh induced by clindamycin. In fact, when comparing the responses to ACh between the Clin group and Clin+F1 or Clin+F2, no significant differences were observed (Fig. 2).

Effects on the mRNA expression levels of TLR receptors

Given the close relationship between the microbiota and the intestinal immune system, changes in the composition of the microbiota can alter the expression pattern of TLRs [22]. Therefore, in this study, we analyzed antibiotic-induced changes in TLRs capable of recognizing bacteria. As shown in Fig. 3 clindamycin treatment altered the expression of TLR2 and TLR4 receptors in different and specific ways. Clin significantly reduced the expression of TLR2, and this decrease was reverted in Clin+F1 and Clin+F2 groups. F1 and F2 did not modify the expression levels of TLR2 compared to Control group (Fig. 3B). When comparing expression differences relative to the Clin group,

significant differences were observed in all groups except the Clin+F1 group. All Clin-treated mice showed a significant increase in TLR4 expression compared to Control group, even those that also received F1 or F2, showing that these formulas were not able to revert the decrease induced by Clin in TLR4. F1 and F2 did not significantly modify the expression levels of TLR4 compared to either the Control or Clin group (Fig. 3C). Surprisingly, TLR1 expression was only significantly increased in Clin+F2 group. However, the other clindamycin-treated groups also displayed a slight increase in the expression of this receptor; although, none of these differences were significant compared to the Control group (Fig. 3A). Although TLR5, TLR6, and TLR9 expression levels were not significantly modified by any of the treatments compared to the Control group, a slight decrease in TLR5 and TLR6 receptor expression was observed in the Clin group.(Fig. 3D, E, and F). Clin+F1 and/or Clin+F2 showed a significant increase in TLR5 and TLR6 expression compared to animals treated with clindamycin alone (Fig. 3D and E).

Effects on lipid and protein oxidation

To assess oxidative damage in the colon, we quantified levels of MDA + 4-HDA and carbonyl proteins, which serve as biomarkers indicating lipid and protein oxidative damage, respectively. Clin, F1, and F2 did not modify either MDA + 4-HDA (Fig. 4A) or protein carbonyl levels (Fig. 4B) in the colon of mice. The treatment with F1 or F2 along with Clin also did not modify the oxidative stress levels in the colon (Fig. 4A and B).

DISCUSSION

Gut microbiota is closely related to human health and disease since, among many of its functions, it has been shown to influence the immune system and participate in the maintenance of normal intestinal peristalsis [1,23]. Microbiota and end products of bacterial fermentation promote the synthesis and release of various neurotransmitters [23], which play key roles in modulating intestinal motility via the ENS and the mucosal immune system [11]. An imbalance in the gut microbiota has been established as a cause of motility dysfunction, facilitating the development of functional gastrointestinal disorders [23]. Germ-free animals are frequently utilized to demonstrate the impact of gut microbiota on gut motor function [2], however, broad-spectrum antibiotics can also be used to deplete commensal gut bacteria and to prove the reversibility of microbial effects on intestinal motility [24]. In several types of surgery, intravenous clindamycin is used as preoperative prophylaxis. Clindamycin is excreted through the bile so even if it is given by IP, it will accumulate in the intestine and produces alterations in the microbiota composition [12,25].In

this study, we present evidence that a single IP dose of clindamycin significantly alters intestinal motility. Our results agree with other studies [24,26], in which mice treated with broad-spectrum antibiotics like neomycin and bacitracin showed decreased microbial diversity, slower intestinal transit time, and inhibition of spontaneous colonic contractility. Nevertheless, the mixture of whey or buttermilk supplemented with nLF and MFGM prevented antibiotic-mediated abnormalities in intestinal motility. In the study by Dalziel et al., the ability of whey protein to alter GI motility was demonstrated since whey protein hydrolysate (WPH) acted as a prokinetic promoting motility in rat distal colon [27].

TLRs are known to be responsible for the interaction between gut microbiota and the host. TLR2 and TLR4 receptors have been detected in enteric neurons, glial cells, and smooth muscle cells of the intestinal wall [7,28], suggesting that these TLRs are involved in the regulation of gut motility. In our study, the treatment with clindamycin significantly decreased the expression of TLR2 and increased TLR4 expression in the colon. These findings are in line with previous research conducted in a murine model of clindamycin-induced dysbiosis [12] in which TLR2 expression was decreased in clindamycin-treated mice. In addition, in another study conducted by the same research group, treatment with neomycin and bacitracin also induced a decrease in TLR2 and an increase in TLR4 expression levels [26].

Inhibition of endogenous TLR2 signalling *in vivo* has been associated with dysmotility and loss of colonic myenteric neurons [29]. Moreover, another study showed that mice subjected to antibiotic treatment had anomalies in ENS structure and intestinal contraction, and these defects were partially restored by TLR2 stimulation [7], suggesting that pharmacological approaches based on the promotion of TLR2 signalling might be useful for the treatment of GI motility disorders.

Regarding TLR4, activation of this receptor has been associated with both increased and delayed bowel movement. Anitha et al. reported that $TLR4^{-/-}$ mice were characterized by delayed gastrointestinal motility, a phenotype similar to that observed in germ-free and antibiotic-treated animals [9]. In contrast, another study reported that TLR4 activation by LPS in the ileum of mice reduced the response to ACh [26].

In our study, the animals treated with F1 and F2 along with clindamycin restored TLR2 expression levels similar to Control, suggesting that this receptor may have a role in maintaining intestinal neuromuscular function. Milk proteins in whey and buttermilk possess immunomodulatory potential in innate and acquired immunity. For example, β-LG plays a key role in enhancing immune responses by promoting the proliferation of lamina propria lymphocytes [30]. MFGM proteins have been proven to have an immunomodulatory function, increasing the expression of immune-related genes, such as TLR2 and TLR4, in the colonic mucosa of neonatal piglets [19]. In our last research, we

found that nLF was not able to reverse the clindamycin-induced decrease in TLR2 expression [12]. Nevertheless, in this study, the mixture of whey or buttermilk supplemented with nLF and MFGM restored normal levels of this receptor and prevented antibiotic-mediated abnormalities in intestinal motility.

The antioxidant potential of milk proteins is also well-known and is associated with their amino acid sequence, structure, and molecular weight [31]. In this study, we wanted to test the potential of whey and buttermilk-based formulas to prevent oxidative damage caused by antibiotic treatment. However, we found that a single dose of clindamycin was not able to increase either lipid peroxidation or protein oxidation. This result is in accordance with our previous work [12], in which histological assessment of the colon showed no inflammatory changes in the clindamycin-treated group. Therefore, further research is required to elucidate the antioxidant potential of these milk formulas in an inflammatory situation.

CONCLUSIONS

In conclusion, our results, together with previous studies, confirm that antibiotic treatment leads to disorders in gut microbiota and TLR expression, which can affect the neuromuscular regulation of gastrointestinal motility. Treatment with whey or buttermilk supplemented with native bovine lactoferrin and MFGM restores TLR2 expression levels and clindamycin-induced alterations in intestinal motility. Therefore, it could be interesting to develop functional foods with this combination of bioactive ingredients to treat motility disorders and restore gut health after antibiotic use.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTION STATEMENT

A. Bellés: performed the experiments, carried out the data collection, analysis, and interpretation, and wrote the first draft of the manuscript. I. Abad, C. Vergara, B. Buey and J.E. Mesonero: performed the experiments. L. Sánchez: performed the experiments and was responsible for funding acquisition. L. Grasa: performed the experiments, supervised, reviewed the manuscript, and was responsible for funding acquisition.

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TABLES

Table 1. Primers used for quantification of TLRs in mouse colon by q-RT-PCR

Gene	Reference	GenBank accession number	Forward and reverse primers
TLR1	12	NM_030682	FW: TCTTCGGCACGTTAGCACTG RV: CCAAACCGATCGTAGTGCTGA
TLR2	12	NM_011905	FW: GCCACCATTTCCACGGACT RV: GGCTTCCTCTTGGCCTGG
TLR4	12	NM_021297	FW: AGAAATTCCTGCAGTGGGTCA RV: TCTCTACAGGTGTTGCACATGTCA
TLR5	12	NM_016928	FW: ATGGCATGTCAACTTGACTT RV: GATCCTAAGATTGGGCAGGT
TLR6	12	NM_011604	FW: TCATCTCAGCAAACACCGAGTATAGCG RV: CAACCTTATTGAATGTGACCCTCCAGC
TLR9	12	NM_031178	FW: ACTTCGTCCACCTGTCCAA RV: AGGAAGGTTCTGGGCTCAAT
GAPDH	12	NM_001289726	FW: AACGACCCCTTCATTGAC RV: TCCACGACATACTCAGCAC
HPRT	12	NM_013556.2	FW: CTGGTGAAAAGGACCTCTCGAA RV: CTGAAGTACTCATTATAGTCAAGGGCAT

FIGURE LEGENDS

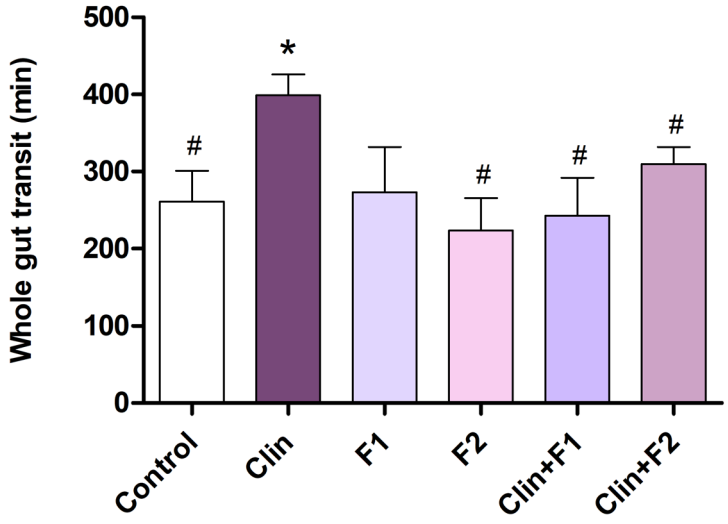

Fig. 1. Whole gastrointestinal transit measured in minutes in the different groups of mice: Control, Clin, F1, F2, Clin+F1, and Clin+F2. Results represent the mean ± SEM of 5 mice per group. * $p<0.05$ versus Control group; # $p<0.05$ versus Clin group.

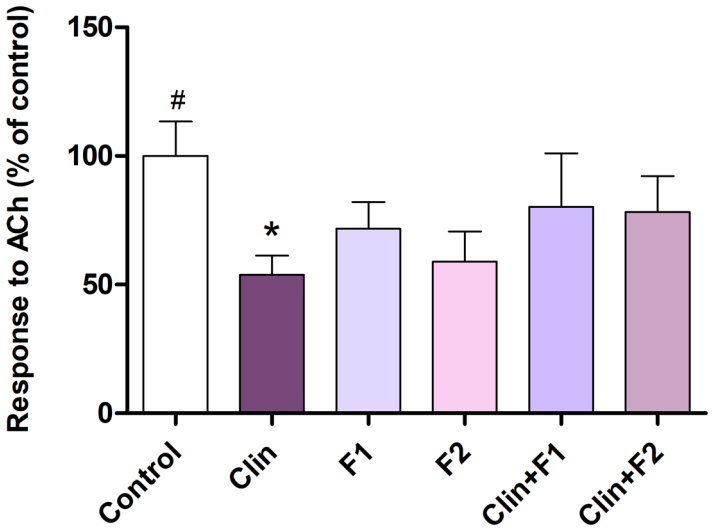

Fig.2. ACh-evoked contractions in ileal longitudinal muscle from Clin, F1, F2, Clin+F1, and Clin+F2 treated mice, expressed as a percentage of the Control group (100%). Results represent the mean ± SEM of 5 mice per group. * $p<0.05$ versus Control group; # $p<0.05$ versus Clin group.

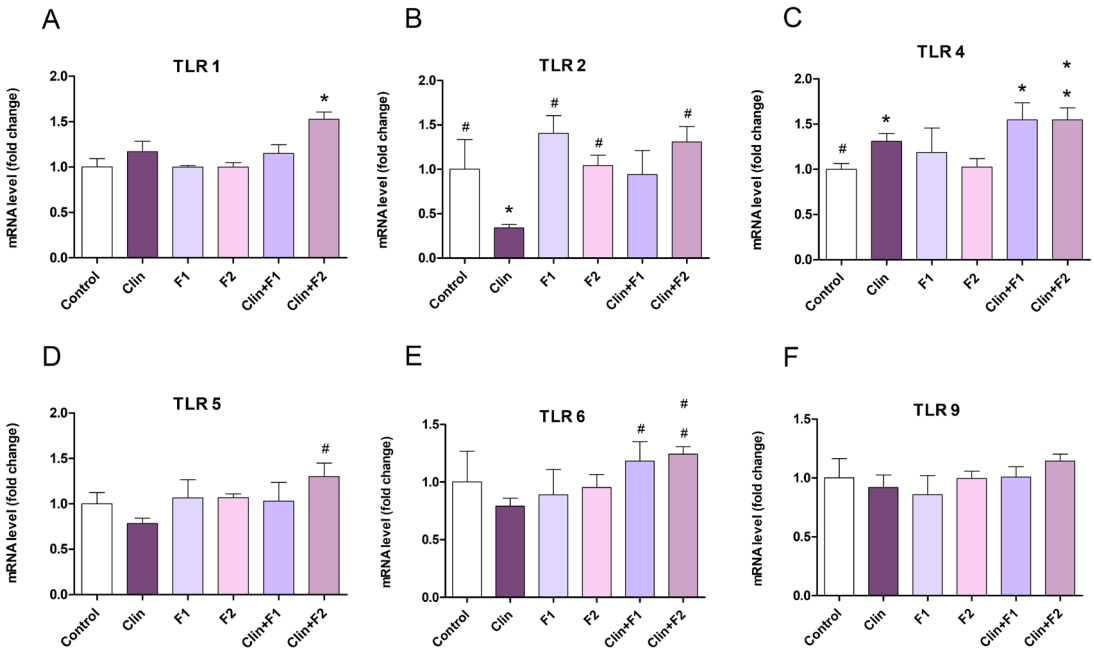

Fig.3. Expression levels of TLR1, TLR2, TLR4-6, and TLR9 mRNA in the colon of Control, Clin, F1, F2, Clin+F1, and Clin+F2 treated mice. The results are presented as fold change compared to Control, with Control normalized to 1. The results represent the mean values ± SEM of 5 mice per group. * $p<0.05$, ** $p<0.01$ versus Control group; # $p<0.05$, ## $p<0.01$ versus Clin group.

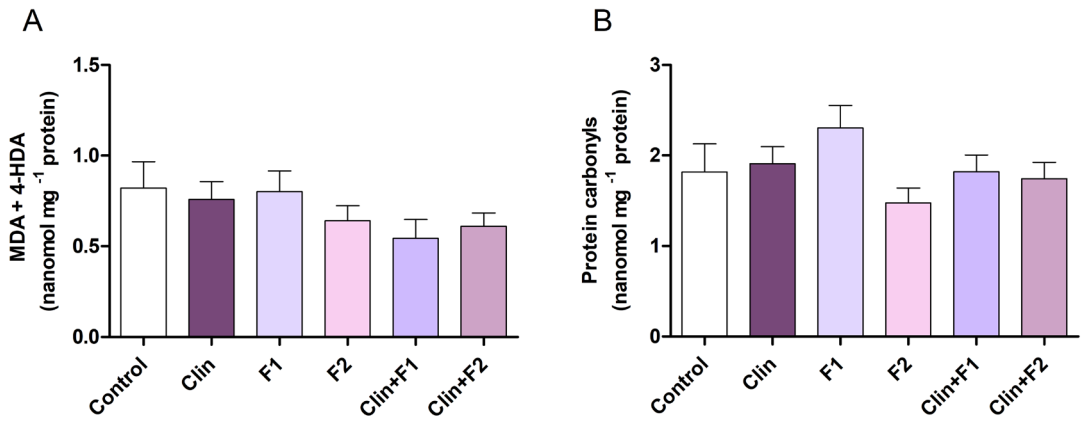

Fig.4. Effect of different treatments (Clin, F1, F2, Clin+F1, and Clin+F2) on lipid peroxidation (A) and protein oxidation (B) in the colon. The results are the mean ± SEM of 5 mice per group.