ARTICLE

Lactoferrin modulates gut microbiota and Toll-like receptors (TLRs) in mice with dysbiosis induced by antibiotics

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Background: Antibiotic administration can result in gut microbiota and immune system alterations that impact health. Bovine lactoferrin is a milk protein with anticancer, anti-inflammatory, antimicrobial and immune modulator activities. The aim was to study the ability of native and iron-saturated lactoferrin to reverse the effects of clindamycin on gut microbiota and intestinal Toll-like receptors (TLRs) expression in a murine model. **Methods:** Male C57BL/6 mice were treated with vehicle, clindamycin (Clin), native bovine lactoferrin (nLf), nLf + clindamycin (nLf_Clin), iron-saturated bovine lactoferrin (sLf) and sLf + clindamycin (sLf_Clin). Fecal samples of each group were collected, and bacterial DNA was extracted. Sequencing of 16s rRNA V4 hypervariable gene regions was conducted to assess the microbial composition. mRNA expression levels of TLRs (1-9) were determined in mice colon by qPCR. Pearson correlation test was carried out between bacteria showing differences in abundance among samples and TLR2, TLR8 and TLR9. **Results**: Beta-diversity analysis showed that the microbial community of the vehicle was different from the communities of Clin, nLf_Clin and sLf_Clin. At the family level, Bacteriodaceae, Prevotellaceae and Rikenellaceae decreased in the Clin group and the treatment with nLf or sLf reverted these effects. Clin reduced the expression of TLR2, TLR8 and TLR9 and sLf reverted the decrease in the expression of these receptors. Finally, TLR8 was positively correlated with Rikenellaceae abundance. **Conclusion:** In a situation of intestinal dysbiosis induced by clindamycin, lactoferrin restores the normal levels of some anti-inflammatory bacteria and TLRs and, therefore, could be a good ingredient to be added to functional foods.

1. Introduction

The human gastrointestinal tract contains an extremely dense and diverse microbial community, known as the gut microbiota. This bacterial community establishes a symbiotic relationship with the host, which is essential for the maintenance of homeostasis and host health, preventing colonization of infectious agents or the overgrowth of commensal species with pathogenic potential. Furthermore, gut bacteria and their products play an important role in the development and modulation of the immune system [1]. In particular, the crosstalk between gut microbiota and toll-like receptors (TLRs), a family of pattern-recognition receptors present on numerous immune and epithelial cells, helps maintain the homeostasis of intestinal immunity [2]. TLRs can recognize microbe-associated molecular patterns (MAMPs, expressed by resident microbiota) and pathogen-associated molecular patterns (PAMPs, expressed by microorganisms with pathogenic potential). In mammals, 13 different TLRs have been identified; some of them are specialized in bacterial recognition such as TLR1, TLR2, TLR4-TLR6 and TLR9. TLR2 recognizes various ligands from Gram-positive and Gram-negative bacteria. TLR4 mainly recognizes lipopolysaccharide (LPS) or endotoxin from Gram-negative bacteria. TLR5 is the receptor for bacterial flagellin and TLR9 recognizes intracellular bacteria. TLR3, TLR7, TLR8 and TLR9 can detect virus nucleic acids [3]. In healthy conditions, activation of these receptors by pathogens induces several intracellular signalling cascades resulting in the production of cytokines, chemokines and transcription factors that trigger an inflammatory response. Conversely, constant exposure to commensal bacteria suppresses the activation of the immune system, thus protecting the mucosa from unnecessary inflammation. Therefore, commensal composition and tolerance are essential mechanisms of maintaining hyporesponsiveness of the intestinal immune system [4, 5].

The intestinal microbiota is established early in life. However, its composition can be shaped by host factors, including age, sex and genetics, and environmental factors such as dietary habits and some drugs [6]. Indeed, alterations in the composition and functionality of the microbiota, known as dysbiosis, are extensively associated with antibiotic intake. Antibiotics can affect gut microbiota through direct or indirect mechanisms. Broad-spectrum antibiotics perturb the original microbiota composition, reducing bacterial diversity while

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increasing and reducing specific taxa [1, 7]. In addition, antibiotics may also indirectly affect the gut microbiota by altering microbial symbiotic relationships in the gut. Thus, loss of specific populations can lead to alteration of the microenvironment in the gut, which in turn affects the growth of other members of the gut microbiota [8].

Besides the effects that antibiotics have on the composition of the gut microbiota, these drugs also affect the way this community interacts with the host through the activation of TLRs. As microbiota composition changes, the altered community will present a different repertoire of MAMPs to the TLRs located on immune cells and intestinal epithelial cells [9]. Depletion of Gram-negative bacteria reduces TLR4-mediated signalling, whereas the depletion of Gram-positive bacteria decreases TLR2 activation [8]. Furthermore, antibiotic-induced dysbiosis not only affects TLRs signalling but also compromises the development and function of immune cells in the gut. Disruption of the gut microbiota by antibiotics has a profound impact on innate lymphoid cell type 3 (ILC3). These cells are well known for their ability to maintain the integrity of the intestinal barrier and to shape the immune response to gut microbiota through an IL-22-dependent pathway [10, 11]. All these changes alter immune homeostasis in the gut and make the host more susceptible to infection by opportunistic pathogens and intestinal infections.

In particular, antibiotic-associated diarrhea (AAD) has been linked with organisms such as *Klebsiella pneumoniae*, *Staphylococcus aureus* and, of most concern, *Clostridium difficile*, which can cause life-threatening diarrhea in hospitalized patients [12]. The highest-risk antibiotics associated with *C. difficile* infection (CDI) include cephalosporins, fluoroquinolones, clindamycin and carbapenems [12]. Indeed, a single dose of clindamycin has been shown to induce profound changes in the composition of the microbiota of mice and, consequently, to confer long-term susceptibility to C. *difficile* infection [13]. Clindamycin is a lincosamide antibiotic with broad-spectrum activity against Gram-positive and anaerobic bacteria. It is excreted in bile and becomes highly concentrated in feces, which has a major negative impact on the intestinal microbiota [13]. Currently, antibiotics and faecal microbiota transplantation (FMT) are the main treatments for CDI [14]. Nevertheless, the use of antibiotics is associated with a greater recurrence of the infection, and FMT can produce adverse effects in immunosuppressed patients [15].

Consequently, it is necessary to develop new strategies to modulate the intestinal microbiota and stimulate the immune system to prevent the side effects of antibiotics. In this context, the diet has been considered one of the major modulators of gut microbiota, thus the search for new functional food ingredients is an interesting field of research.

Bovine lactoferrin (BLf) is an iron-binding glycoprotein that is mainly present in milk but also in most external fluids of mammals, such as saliva, tears or intestinal secretions [16]. This protein displays a wide range of biological properties, including antimicrobial, anti-inflammatory, antioxidant and immunomodulatory activities [16, 17]. Moreover, several *in vitro* studies have shown that lactoferrin, due to its ability to bind iron, can limit the growth of pathogenic bacteria and enhance the growth of specific probiotics that can grow under iron-deficient conditions, such as *Lactobacillus* and *Bifidobacterium* spp. [16, 18]. Therefore, lactoferrin could contribute to the maintenance of intestinal homeostasis and counteract the dysbiosis induced by antibiotic treatment.

The structure of lactoferrin consists of a single polypeptide chain organized in two homologous lobes, each responsible for the reversible binding of a single ferric atom. Depending on the iron-saturation level, lactoferrin (Lf) exists in three different forms: iron-depleted Lf (apoLf), monoferric Lf and iron-saturated Lf (holoLf) [19]. Native-lactoferrin (nLf) obtained from bovine milk is characterized by an iron saturation level of 10-15%. The influence of Lf iron saturation on its biological activity is not completely known, as it depends on the evaluated activity and some environmental factors. Thus, nLf shows antibacterial activity against several foodborne bacteria, while holoLf does not have any activity [17, 20]. However, the same antiviral activity against rotavirus has been observed for bovine Lf, in both native and holo forms [21]. Several assays have also shown that, depending on iron saturation, Lf displays divergent effects on the growth of probiotic bacteria [17]. The capacity of bovine Lf to modulate positively the gut microbiota has been previously demonstrated in high-fat diet induced obese mice [22], dextran sulfate sodium induced colitis model [23] or Alzheimer´s disease models [24]. However, its potential to counteract the side effects of antibiotics on the microbiota and the immune system has not yet been fully studied.

Therefore, this study aimed to investigate the effects of the antibiotic clindamycin on mouse gut microbiota and TLR receptor expression and the relationship between TLRs and the dysbiosis status. In addition, we evaluated the prebiotic potential of native and saturated lactoferrin to reverse these effects.

2. Materials and Methods

2.1. Native and iron-saturated bovine lactoferrin (bLf) solutions preparation

Native bLf (iron-saturation below 10%) was kindly provided by Tatua Nutritionals Company (Morrinsville, New Zealand). SDS-PAGE quality analysis showed a single band corresponding to a protein of about 80 KDa and purity higher than 90%. bLf stock solution was prepared from the native protein in saline at a concentration of 200 mg/ml and sterilized using low-binding protein 0.22 µm filters. After filtration, the absorbance was measured at 280 nm and the concentration of bLf was determined by considering a molar extinction coefficient ($E^{1\%}$) of 1.27 ml/cm/g. Native bLF was saturated with iron by adding ferrinitrilotriacetate (FeNTA) solution as described previously [25]. Afterwards, bLF was subjected to Sephadex G-25 chromatography to remove unbound iron. The iron-saturated bLF solution was filtered using 0.22 µm filters and the concentration was determined, considering a $E^{1\%}$ of 1.51 ml/cm/g. The final concentration of native and iron-saturated bLf was adjusted to 175 mg/ml.

2.2. Animal model and provided treatments

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. The study was carried out from two independent groups (experiments 1 and 2, at different times) of 30 male C57BL/6 mice (8-12 weeks old, Janvier Labs, Le Genest-Saint-Isle, France), a total of 60 mice. They were kept in a conventional laboratory animal facility at the University of Zaragoza at a range of temperatures between 20–22 °C, in a 12-h light/dark cycle, with free access to animal chow and water. To normalize gut microbiota mice colonies of all groups tested were treated equally: housed in the same room, kept in the same cages and maintained by the same personnel. For each experiment, mice were randomly divided into 6 groups (n=5 per group): vehicle (they received saline orally by gastric gavage for 10 days and they represent the 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). Mice treated with clindamycin were gavaged for 10 days with saline and on day 4 received a single IP injection of 200 µg of clindamycin (Normon Laboratories, Madrid, Spain) diluted in 200 µl of saline. Mice from the groups nLf and sLf were treated for 10 days orally by gastric gavage with 35 mg nLf or sLf, diluted in 200 µl of saline. Mice from nLf_Clin and sLf_Clin groups were gavaged for 10 days with 35 mg nLf or sLf and on day 4 received an IP injection of 200 µg of clindamycin. Intestinal dysbiosis caused by clindamycin has been previously described [13], and similar doses of lactoferrin have shown to have effects on the intestinal immune system of mice [26, 27]

2.3. Bacterial DNA extraction

Following each treatment, stool samples were collected in soil grinding sterile SK38 2 ml tubes (Bertin Technologies, Montigny-le-Bretonneux, France). Samples were stored at −80°C until processed. Bacterial DNA was extracted from frozen samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the protocol before described [28]. Two mouse fecal pellets were mixed with 1 ml InhibitEX buffer in SK38 tubes and processed by using the Precellys® 24 homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) for 2 x 30 sec at 6,500 rpm and 10 sec delay between cycles. Lysis was completed at 95°C for 5 min. Finally, DNA was extracted according to the instruction of the QIAamp Fast DNA Stool Mini Kit and eluted in 40 µl elution ATE buffer. DNA concentrations were measured with Qubit® 4.0 fluorometer (Invitrogen, Life Technologies, Madrid, Spain) and dsDNA HS (high sensitivity) Assay Kit (Invitrogen), following the manufacturer's instructions. DNA purity was assessed by measuring the A260/A280 with NanoDrop® ND-1000 Spectrophotometer V3.0.1 (Thermo Fisher Scientific, Madrid, Spain) and monitored on 1% agarose gels.

2.4. Sequencing of bacterial 16S rRNA gene

According to the concentration, DNA was diluted to 1 ng/µl using sterile water. 16S rRNA gene of V4 region was amplified using specific primer (515F-806R) [29] with a barcode. All PCR reactions were performed with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA). The same volume of 1 x loading buffer (contained SYBR green) was mixed with PCR products and amplicons were detected by electrophoresis on 2% agarose gel. Samples with bright main strip between 400-450 bp were chosen for further experiments. PCR products were mixed in equidensity ratios. Then, the mixture of PCR products was purified with Qiagen Gel Extraction Kit (Qiagen). Sequencing libraries were generated using NEBNext Ultra DNA Library Pre® Kit for Illumina® (New England Biolabs), following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Invitrogen) and Agilent Bioanalyzer 2100 system (Agilent, Santa Clara, CA, USA). Later, the library was sequenced on an Illumina MiSeq platform and 250 bp paired-end reads were generated. Demultiplexing process to sort the sequenced reads into separate files was conducted at the end. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. The data of the sequences are available in NCBI Sequence Read Archive (SRA), (BioProject ID PRJNA800685).

2.5. Bioinformatic analysis

2.5.1. Reads taxonomic assignment. Analysis of the samples was carried out using QIIME 2 v.2020.2 [30]. Quality filtering on the raw demultiplexed imported sequences was performed under specific filtering conditions to obtain high-quality clean sequences according to DADA2 method [31] available in QIIME 2. Sequences with ≥ 97% similarity were clustered and assigned to an amplicon sequence variant (ASV). To identify the bacteria present in the samples, we aligned sequences using SILVA138_97 [32] as a reference database. The Silva 138_97 sequence reference database was formatted, managed and manipulated using RESCRIPt QIIME 2 plugin [33] to obtain the reference sequences and taxonomy files. The Naive Bayer classifier was trained using the reference sequences and taxonomy. Sequence classification was performed using the trained classifier and the machine-learning-based classification methods available in scikit-learn 0.22.1 through the classify-sklearn QIIME 2 plugin.

2.5.2. Phylogeny. Calculated phylogeny was used later in the downstream analyses for diversity. First, the MAFFT algorithm (Multiple Alignment using Fast Fourier Transform) [34] from QIIME 2 was used to perform a multiple sequence alignment of the sequences. Next, the alignment was masked or filtered to remove highly variable positions and FastTree2 [35] was used to generate a phylogenetic tree.

2.5.3. Bacterial composition. ASV tables, metadata data frame, taxonomy table and the rooted-tree were imported to R software [36], using qiime2R [37] to get a phyloseq object. The bacterial composition was determined by using the phyloseq R package [38]. The differential abundance of bacteria, at phylum and family levels, between each pair of groups was compared by using the Wilcoxon rank test ($p < 0.05$).

2.5.4. Diversity calculation. ASV abundance information was normalized dividing by the total number of sequences in the sample that has fewer sequences. Rarefaction curves and alpha and beta diversity were determined by phyloseq and vegan R packages [38, 39]. Rarefaction, to a subsampling depth (determined by the minimum number of sequences for each experiment) and step size of 50 for each iteration, was performed on all samples to standardize the sequencing effort. To study the alpha diversity, we calculated and represented in a graph the observed ASVs and Shannon index for each group of samples. The changes in the observed ASVs and Shannon index between the different groups were analyzed using the Wilcoxon test ($p < 0.05$). To study the beta diversity, a multivariate cluster analysis based on Bray-Curtis distance was performed and a Non-metric Multidimensional Scaling (NMDS) was represented to show the dissimilarity between groups. A PERMANOVA test was performed to see if there were significant differences between each pair of groups (999 permutations, $p < 0.05$).

2.5.5. Pearson correlation test. A Pearson correlation test was carried out between bacteria showing differences in abundance among all samples and the three most relevant TLR: TRL2, TRL8 and TRL9 using the cor function by default parameters from stats package in R. Results were plotted with R package 'corrplot' [40]. Statistical significance results for each correlation test were considered with P values <0.05.

2.6. TLR expression in mice colon by qPCR

Following each treatment, mice were euthanized by cervical dislocation, the colon was excised, and proximal colon samples were preserved in RNAlater solution (Ambion, Thermo Fisher Scientific, Madrid, Spain) for 24 hours and then stored at -80 °C. Total RNA was isolated using RNeasy mini Kit (Qiagen), following the manufacturer's instructions. cDNA was generated from RNA using qScrip cDNA SuperMix kit (Quantabio, Beverly, MA, USA) according to the supplier's protocol. cDNAs obtained by reverse transcription were used to determine mRNA expression levels of TLRs. Real-time PCR was performed on the StepOnePlus Real-Time PCR System (Life Technologies, Carlsband, CA, USA), using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and specific primers of Table 1. Each sample was run in triplicate and the mean Ct was determined from the three runs. The relative mRNA expression under each experimental condition was expressed as $\Delta Ct = Ct_{gene} - Ct_{calibrators}$, with GAPDH and HPRT as housekeeping genes. Then, the relative gene expression levels were calculated as $\Delta\Delta Ct = \Delta Ct_{control} - \Delta Ct_{treatment}$, and finally converted and expressed as fold difference ($2^{-\Delta\Delta Ct}$). The Mann-Whitney U test was used to compare mRNA expression of TLRs in mice intestine through software GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA) and the differences between P values <0.05 were considered to be statistically significant.

2.7. Microscopic Assessment: Histology

Samples of colon were fixed in 10 % neutral buffered formalin, dehydrated, and paraffin-embedded. Four-micrometer sections were stained with hematoxylin and eosin, and coded slides were evaluated by two persons unaware of the treatments to prevent observer bias.

3. Results

3.1. Raw sequence processing to obtain the ASVs

The minimum and the maximum number of sequences present in the samples were 111,348 and 608,374, being the mean 244,463 sequences. The maximum percentage of chimeric sequences found in the samples was 17%, indicating the good performance of the sequencing. Experimental measurements of chimera formation during PCR amplification of 16S rRNA sequences from mixed bacterial genomic DNA have indicated chimera formation rates of over 30% [41]. Only one sample of the two experiments showed chimera formation rates over 30% and was discarded from the study.

Rarefaction curves of experiments 1 and 2 showed that libraries were large enough to cover most of the bacterial diversity in each sample (Figure S1. In the rarefaction curves of both experiments, a maximum diversity saturation of ASVs is observed with 10,000 sequences and is accentuated with 30,000 sequences. In experiment 1, the number of different ASVs observed in most samples was in the range of 400-800, while in experiment 2 was 150-300, indicating a lower diversity in the samples of experiment 2 respect to experiment 1 (Figure S1).

3.2. Bacterial composition

The bar-plots representing the bacterial composition at the phylum level for all 59 samples are shown in Figure 1. We observed that most sequences were assigned to Firmicutes and Bacteroidota. The Verrucomicrobiota, Proteobacteria and Actinobacteriota phyla were also present in all groups of treatment, although in lower proportions. Most of the Bacteroidota sequences belonged to the Bacteroidales order, being Muribaculaceae (20%), Rikenellaceae (6%), Tannerellaceae (2.5%), Prevotellaceae (1%), Bacteriodaceae (1%) and Marinifilaceae (0.8%) the most abundant familiesMost of the Firmicutes sequences belonged to the Lachnospirales and Oscillospirales orders, being Lachnospiraceae (20%) and Oscillospiraceae (3%) the most abundant families. Other less abundant families found between the Firmicutes were the Clostridia, Butyricoccaceae, Ruminococcaceae and Anaerovoracaceae.

Table 1. Primers used for quantification of TLRs in mouse colon by qPCR.

Gene	Reference	GenBank accession number	Sense and antisense primers	Size (bp)
TLR1	[42]	NM_030682	FW: TCTTCGGCACGTTAGCACTG RV: CCAAACCGATCGTAGTGCTGA	100
TLR2	[42]	NM_011905	FW: GCCACCATTTCCACGGACT RV: GGCTTCCTCTTGGCCTGG	66
TLR3	[42]	NM_126166	FW: GGTCCCCAGCCTTCAAAGAC RV: ACGAAGAGGGCGGAAAGGT	84
TLR4	[42]	NM_021297	FW: AGAAATTCCTGCAGTGGGTCA RV: TCTCTACAGGTGTTGCACATGTCA	80
TLR5	[42]	NM_016928	FW: ATGGCATGTCAACTTGACTT RV: GATCCTAAGATTGGGCAGGT	299
TLR6	[42]	NM_011604	FW: CAACCTTATTGAATGTGACCCTCCAGC RV: TCATCTCAGCAAACACCGAGTATAGCG	248
TLR7	[42]	NM_133211	FW: GTACCAAGAGGCTGCAGATTAGAC RV: AGCCTCAAGGCTCAGAAGATG	136
TLR8	[42]	NM_133212	FW: GAAGCATTTCGAGCATCTCC RV: GAAGACGATTTCGCCAAGAG	138
TLR9	[42]	NM_031178	FW: ACTTCGTCCACCTGTCCAA RV: AGGAAGGTTCTGGGCTCAAT	114
GAPDH	[42]	NM_001289726.1	FW: AACGACCCCTTCATTGAC RV: TCCACGACATACTCAGCAC	190
HPRT	[43]	NM_013556.2	FW: CTGGTGAAAAGGACCTCTCGAA RV: CTGAAGTACTCATTATAGTCAAGGGCAT	109

All Verrucomicrobiota sequences belonged to the Akkermansiaceae family (2%). All Proteobacteria sequences belonged to the Rhodospirillales order. All the Actinobacteriota sequences belonged to the Coriobacteriales order, being Atopobiaceae and Eggerthelaceae the principal families (Figures S2 and S3).

3.3. Alpha and Beta diversity

We analyzed the bacterial communities of the groups of both experiments in terms of alpha-diversity, by using the observed species (count of unique species) and Shannon index estimators (Figure 2). The number of observed species was lower in all groups of experiment 2 respect to experiment 1, corroborating the results observed in the rarefaction curves. The Shannon index was in the range of 3.5-5 in the groups of both experiments, indicating the well-known high diversity of the gut bacterial community. No differences were found in the Shannon index of the groups of experiment 1 (Figure 2 A). However, in experiment 2, the Shannon indexes in nLf, nLf_Clin and sLf_Clin groups were statistically lower than the Vehicle group (Figure 2 B).

Gut microbiome profiles were clustered using the Bray-Curtis dissimilarity approach and ordinated by using the non-metric multidimensional scaling (NMDS) method. NMDS indicated a distinct clustering of microbiota community for each treatment group in each experiment, implying that different treatments led to the development of various gut microbial communities (Figures 3, 4 and S4). Similarly, when comparing global community patterns (beta-diversity) via Adonis models adjusted for the group of treatment, we found significant differences between the groups, based on Bray-Curtis distance (PERMANOVA, 999 permutations). In both experiments, the microbial community of the Vehicle group was statistically different from the communities of Clin, nLf_Clin and sLf_Clin groups (Figures 3 A, D, E and 4 A, D, E). While in experiment 2 we found differences between the bacterial communities of nLf

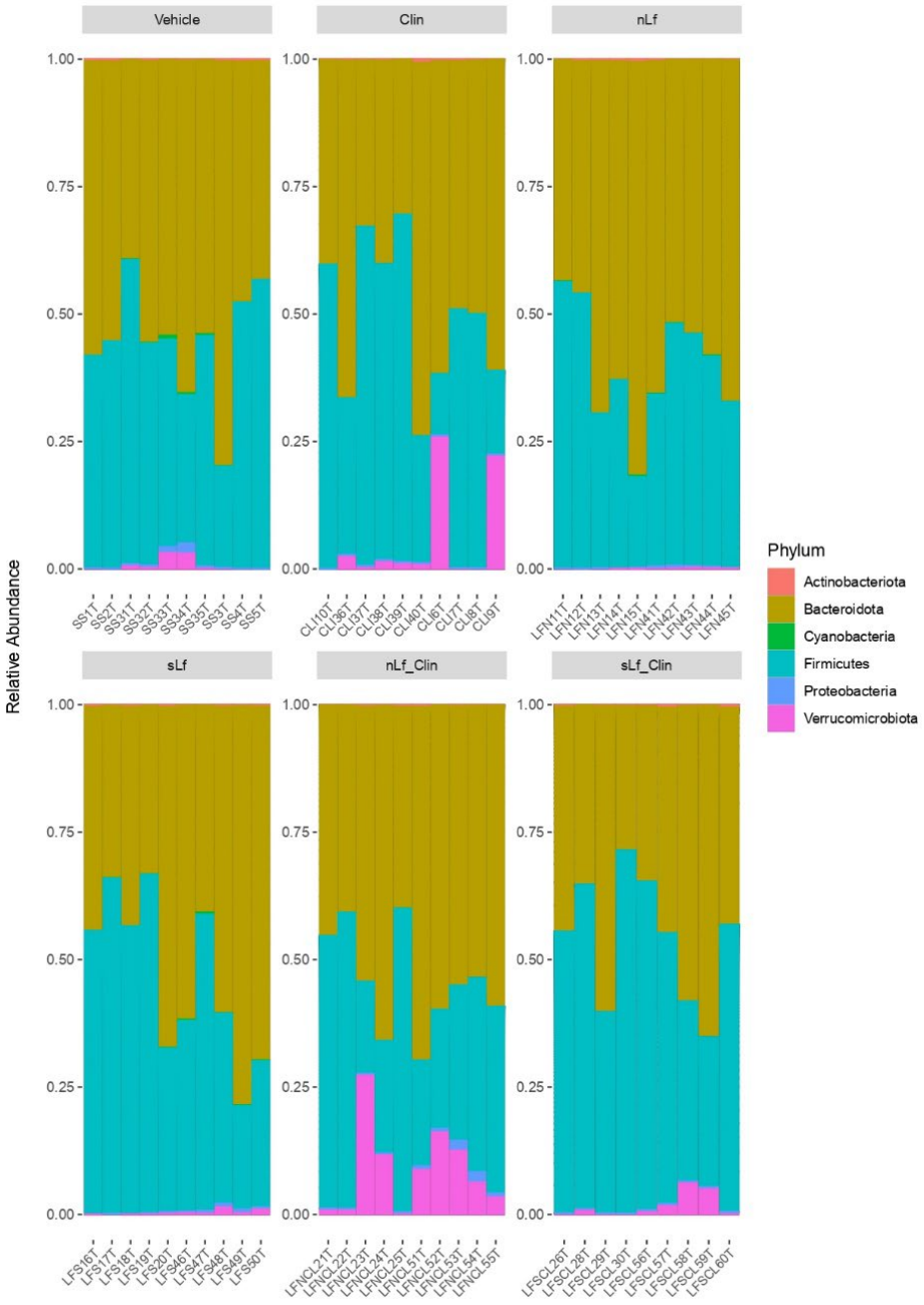

Figure 1. Bacterial relative abundances at phylum level in each sample of vehicle (Control, labeled with SS), clin (labeled with CLI), nLf (labeled with LFN), sLf (labeled with LFS), nLf_Clin (labeled with LFNCL) and sLf_Clin (labeled with LFSCL) groups.

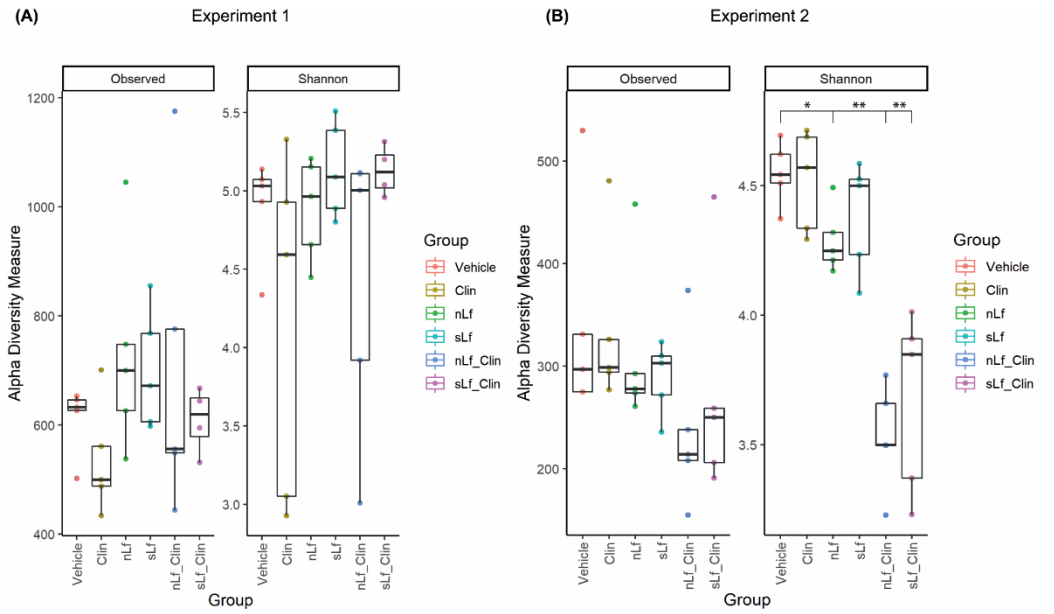

Figure 1. Observed species and Shannon index alpha-diversity estimators across the different groups of treatment of experiment 1(A) and experiment 2(B).

and sLf groups (Figures 4 B, C), these differences were not statistically significant in experiment 1 (Figures 3 B, C). There were no differences between the bacterial communities of Clin, nLf_Clin or nLf_Clin (Figure 3 F). In addition, there were also differences between the bacterial communities of Clin, nLf_Clin or nLf_Clin groups of experiment 2 (Figure 4 F), but not in experiment 1 (Figure 3 F).

3.4. Differences in bacterial abundance

We further analyzed the differences in the abundance of bacteria at the phylum and family level in the different groups respect to Vehicle, by using the Wilcoxon test (Figure 5). No differences in the phyla Actinobacteriota, Bacteroidota, Firmicutes and Proteobacteria were observed between Vehicle and Clin, nLf, sLf, nLf_Clin or sLf_Clin groups. However, the phylum Cyanobacteria was reduced in the groups treated with Clin, nLf_Clin or sLF_Clin respect to Vehicle. Verrucomicrobiota was increased in nLf_Clin group respect to Vehicle (Figure 5).

When we analyzed the abundance of bacteria at the family level, we found that Bacteriodaceae, Marinifilaceae, Prevotellaceae, Rikenellaceae (belonging to Bacteroidota phylum, Figures 6 A-D), Clostridia UCG-014, Ethanoligeneaceae (belonging to Firmicutes phylum, Figures 6 G, H) and Gastranaerophilales (belonging to Cyanobacteria phylum, Figure 6 F) were decreased in the mice treated with clindamycin. The treatment with nLf and/or sLf along with clindamycin reverted these effects, increasing the levels of the bacteria of all these families except for Marinifilaceae and Gastranaerophilales. The clindamycin treatment evoked an increase in Tannerellaceae bacteria and this effect could not be attenuated by nLf or sLf (Figure 6 E).

3.5. Expression of TLRs

As shown in Figure 7, clindamycin treatment reduced the expression of TLR2, TLR8 and TLR9 and the treatment with sLf_Clin but not with nLf_Clin reverted the decrease in the expression of these receptors. Both nLf and sLf did not modify the expression levels of TLR2 but induced a significant reduction in TLR8 expression. Only the nLf was able to reduce per se the TLR9 expression levels. TLR5 expression was significantly reduced in mice treated with nLf and TLR7 expression was significantly increased in sLf_Clin group. The expression of TLR1, TLR3, TLR4 and TLR6 was not modified by any of the treatments.

3.6. Correlation between TLR expression and microbial changes

A correlation matrix was generated to illustrate how TLR2, TLR8 and TLR9 correlated with families whose abundance was different between groups, and to identify associations between these families (Figure 8). The family Rikenellaceae was positively correlated with the abundance of Bacteroidaceae and negatively correlated with Prevotellaceae. The families Gastranaerophilales, Clostridia_UEG.014 and Ethanoligenenaceae were positively correlated with them and with the family Marinifilaceae. By contrast, Ethanoligenenaceae was negatively correlated with Tannerellaceae. Regarding TLR expression, TLR2 and TLR9 did not show a significant correlation with any bacterial family. However, TLR8 was positively correlated with Rikenellaceae abundance.

3.7. Histopathological analysis

Histological assessment of the colon showed no inflammatory changes or differences between the animals treated with Vehicle (Control), Clin, nLf, sLf, nLf_Clin or sLf_Clin (Figure S5).

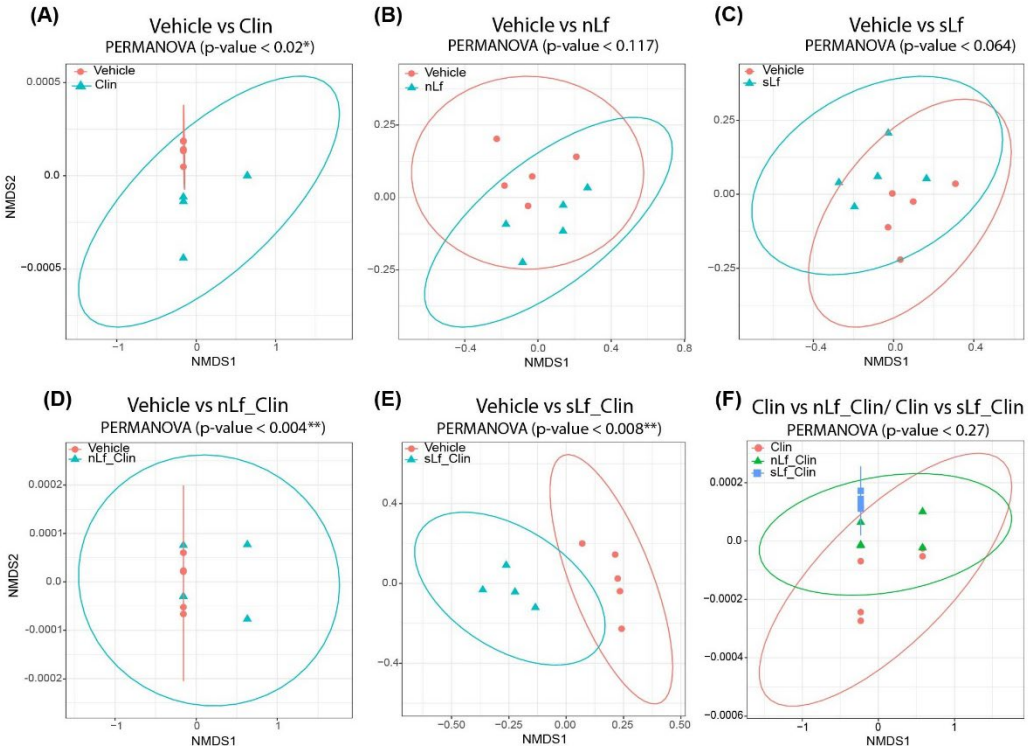

Figure 3. NMDS showing the Bray-Curtis distances between the different microbial populations of the groups Vehicle (Control) vs Clin (A), Vehicle vs nLf (B), Vehicle vs sLf (C), Vehicle vs nLf_Clin (D), Vehicle vs sLf_Clin (E) and Clin vs nLf_Clin vs sLf_Clin (F) in experiment 1.

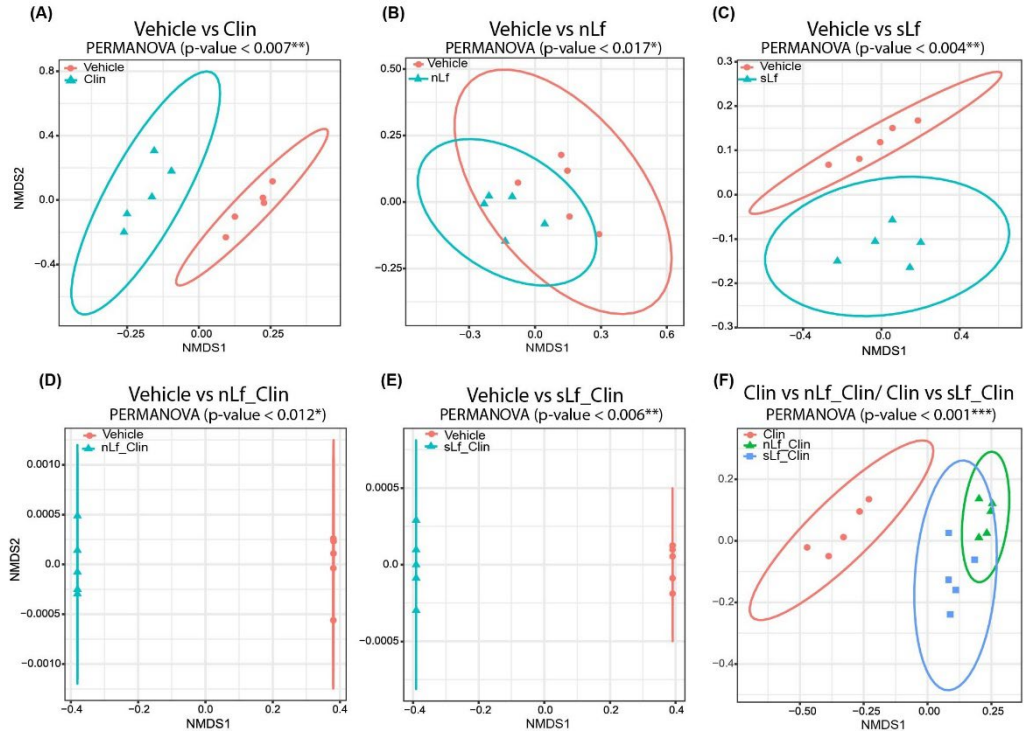

Figure 4. NMDS showing the Bray-Curtis distances between the different microbial populations of the groups Vehicle (Control) vs Clin (A), Vehicle vs nLf (B), Vehicle vs sLf (C), Vehicle vs nLf_Clin (D), Vehicle vs sLf_Clin (E) and Clin vs nLf_Clin vs sLf_Clin (F) in experiment 2.

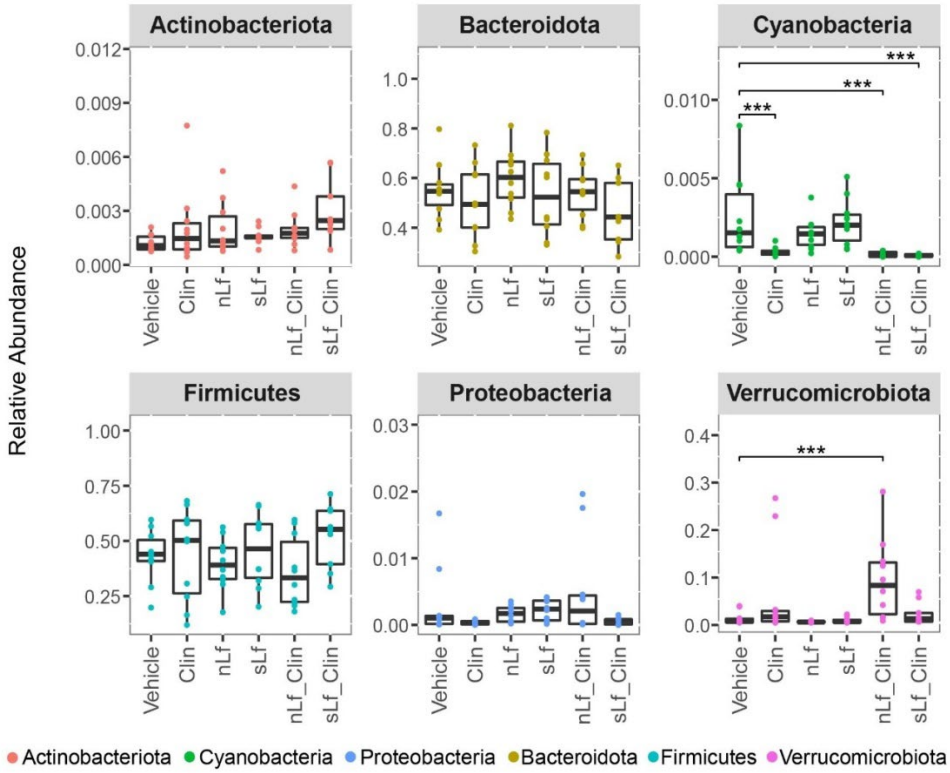

Figure 5. Actinobacteriota, Bacteroidota, Cyanobacteria, Firmicutes, Proteobacteria and Verrucomicrobiota relative abundance in Vehicle (Control), Clin, nLf, sLf, nLf_Clin or sLf_Clin.

4. Discussion

In this study, we used a murine model of clindamycin-induced dysbiosis to determine the potential of nLf and sLf to reverse the effects on intestinal microbiota and TLR expression induced by the antibiotic treatment. Regarding the bacterial composition of the samples, most of the sequences were assigned to the Firmicutes and Bacteroidota phyla, which have repeatedly been described as major and functionally significant components of the human and mouse intestinal microbiota [44, 45]. The Verrucomicrobiota, Proteobacteria and Actinobacteriota phyla were also present, although in lower proportions. When we analyzed the bacterial communities in terms of alpha-diversity, we found that the number of observed species was lower in all groups of experiment 2 respect to experiment 1. These differences may be because the experiments were carried out at different times and the animals were housed in different cages [46]. The Shannon index was in the range of 3.5-5 in the groups of both experiments, similar to the values found by other authors in C57/BL6 male mice [47] . Although the number of observed species and the Shannon index was similar in Vehicle and Clin groups, the beta-diversity analysis showed significant differences between the microbial communities of Clin, respect to Vehicle group in both experiments. Our results agree in part with other studies showing that a single dose of clindamycin results in a dramatic loss in microbiota diversity and alteration in the microbiota composition in mice cecum [13]. According to other studies, clindamycin strongly decreases the abundance of bacteria belonging to Bacteroidota phylum [45, 48, 49]. We found that bacteria from families Bacteriodaceae, Prevotellaceae and Rikenellaceae (belonging to Bacteroidota phylum) were decreased in Clin treated mice, but the treatment with nLf or sLf along with Clin reverted these effects, increasing the levels of the bacteria of all these families.

Bacteroidaceae family, to which the genus *Bacteroides* belongs, includes many important opportunistic pathogens, but as essential members of a balanced microbiota, they are considered health-maintaining. This is due to their ability to reinforce the intestinal epithelial barrier and reduce inflammation by producing anti-inflammatory molecules such as polysaccharide A [50]. Patients with Inflammatory Bowel Disease have shown an increased abundance of *Bacteroides* spp. [51].

Generally, the role of members of the *Prevotella* genus within the intestinal microbiota and their effects on the host is not completely understood and somewhat conflicting interpretations have been reported [52]. High relative abundance of *Prevotella* spp. is found in populations with plant-rich diets [53]. Moreover, it has been shown that *Prevotella* spp. can improve glucose metabolism stimulated by the intake of prebiotics [54]. Together, these studies suggest that *Prevotella* spp. are beneficial microbes. In contrast, other studies have associated *Prevotella* spp. with gut inflammation [55]. Intestinal colonization by *Prevotella* spp. leads to a reduction in short-chain fatty acids, specifically acetate, which reduces IL-18 production and consequently exacerbates intestinal inflammation [52] .

Members of the family Rikenellaceae are hydrogen-producing bacteria that selectively neutralize cytotoxic reactive oxygen

species and protect cells from oxidative stress [56]. In the inflammation process, H_2 mediates the suppression of pro-inflammatory cytokines in inflammatory tissues [57].

Bacteria from families Clostridia UCG-014 and Ethanoligeneaceae (belonging to Firmicutes phylum) were also decreased in the presence of Clin and in this case, this effect was reverted by sLf, but not by nLf.

The family Clostridia UCG-014, previously included in *Ruminococcacea UCG-014* genus, is linked to the formation of butyrate [58]. Butyrate is an energy source for colonocytes that increases colonic mucus secretion and can regulate stem cell proliferation and anti-inflammatory macrophage polarization [59]. The family Ethanoligeneaceae, previously included in Ruminococcaceae family and *Phocea* genus, includes H_2-producing bacteria and some *Clostridium* spp. [32].

Therefore, our studies show that both nLf and sLf are able to maintain stable levels of certain anti-inflammatory bacteria, responsible for maintaining intestinal health.

It has to be taken into account that the level of Lf iron saturation can have different influence on the bacterial growth. On the one hand, nLf can sequester environmental iron, thus limiting the growth of some bacteria, as it is an essential element for them. On the other hand, certain bacteria have developed mechanisms for capturing iron from sLf, using it for their metabolism [60]. In the study by Majka et al.[61] Iron-depleted or iron-saturated Lf did not induce a pro-inflammatory response in a human intestinal cell line or in human and murine macrophages. However, iron-devoid Lf was the most effective form by inhibiting the LPS-dependent activation of murine macrophages.

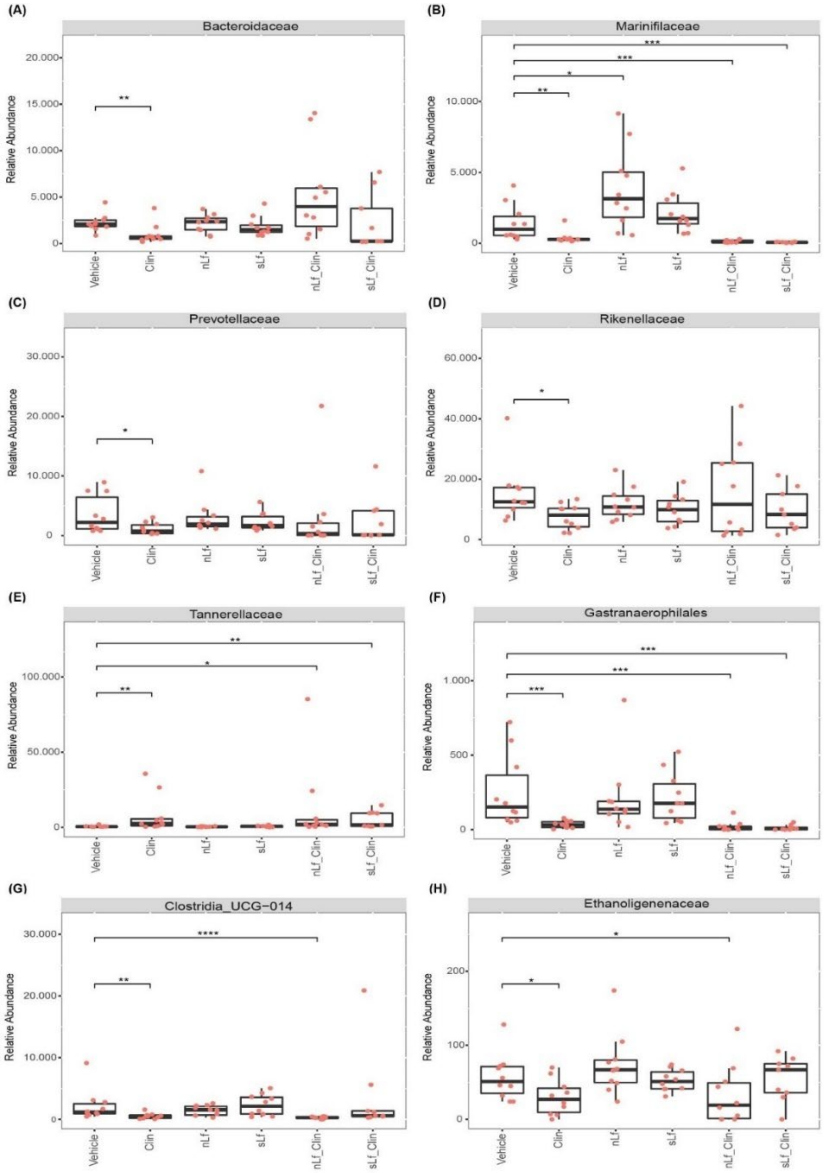

Figure 6. Bacteriodaceae (A), Marinifilaceae (B), Prevotellaceae (C), Rikenellaceae (D), Tannerellaceae (E), Gastranaerophilales (F), Clostridia UCG-014 (G) and Ethanoligenenaceae (H) family relative abundance in Vehicle, Clin, nLf, sLf, nLf_Clin or sLf_Clin groups.

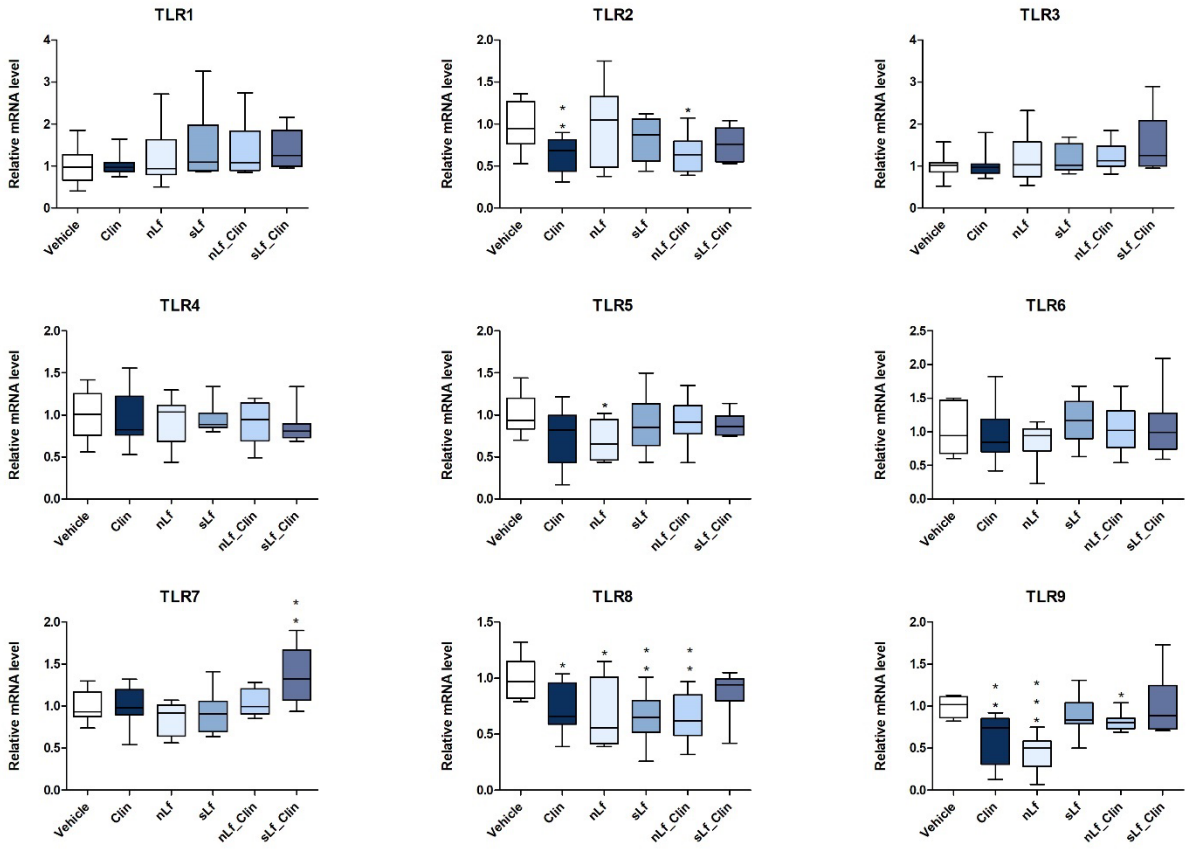

Figure 7. Expression levels of TLR1-9 mRNA in the colon of Vehicle, Clin, nLf, sLF, nLf_Clin and sLf_Clin treated mice. The horizontal line in the middle of each box represents the median, while the top and bottom borders of the box represent the 75 and 25 % percentiles, respectively. Significant differences compared to Vehicle group are indicated by *p<0.05; **p<0.01; and ***p<0.001, n = 10 for each group.

In contrast, Clin treatment evoked an increase in the family Tannerellaceae and a decrease in Marinifilaceae (belonging to Bacteroidota phylum) and Gastranaerophilales (belonging to Cyanobacteria phylum) bacteria, but these effects could not be attenuated by nLf or sLf.

The family Tannerellaceae includes *Parabacteroides* and *Tannerella* genera. In ulcerative colitis patients, Tannerellaceae is decreased in abundance [55]. *Parabacteroides* isolates have shown anti-inflammatory and epithelium reinforcing properties and are considered candidates for bacteriotherapeutic applications aiming to restore gut homeostasis [50, 62].

The family Marinifilaceae includes *Butyricimonas* and *Odoribacter* genera. Decreased levels of *Butyricimonas* are found in chronic social defeat stress-susceptible mice and altered levels of *Butyricimonas* were reported in major depressive disorder patients and untreated multiple sclerosis patients. *Butyricimonas* produce butyrate, which reduces inflammation and helps maintain a healthy gut. Therefore, decreased levels of *Butyricimonas* in the gut may play an inflammation-based role in the pathogenesis of depression [63]. Loss of *Odoribacter* spp. results in reduced short-chain fatty acids (SCFA) availability, leading to intestinal inflammation [64]. Additionally, *Odoribacter* spp. can help maintain lower systolic blood pressure in pregnant women through their production of butyrate. The presence of butyrate reduces blood pressure and has been used in drugs for hypertension [65].

The Gastranaerophilales are found in human and other animal guts and although their exact role is unknown, they are thought to have a beneficial effect for their hosts by aiding digestion and as a source of vitamins B and K [66].

Our studies indicate that Clin increases the levels of the Tannerellaceae family with anti-inflammatory properties and this positive effect is not modified by nLf or sLf. However, the lowering of levels of butyrate-producing bacteria and bacteria that synthesize vitamins and aid digestion evoked by Clin is not reversed by nLf or sLf.

The results of this study have shown that clindamycin decreases the expression of TLR2, TLR8 and TLR9, indicating that the host-microbiota interactions might be also altered. Previously, other authors have described the modulation of TLR expression by antibiotics. The combination of bacitracin and neomycin increased the expression of TLR3, TLR4, TLR6, TLR7 and TLR8 and decreased the expression of TLR2 and TLR9 in the mouse colon [42]. Lincomycin reduced the expression of TLR2, TLR3 and TLR4 in mouse jejunum [67]. The reduction in TLR2 expression induced by antibiotic treatment shown in those studies is consistent with our results. TLR2-dependent mechanisms promote intestinal epithelial cell wound healing and limit pro-inflammatory immune responses during acute and chronic murine colitis [68]. In addition, TLR2 ligands induce IL-10 production and reduce the production of proinflammatory cytokine IL-8 [3, 69]

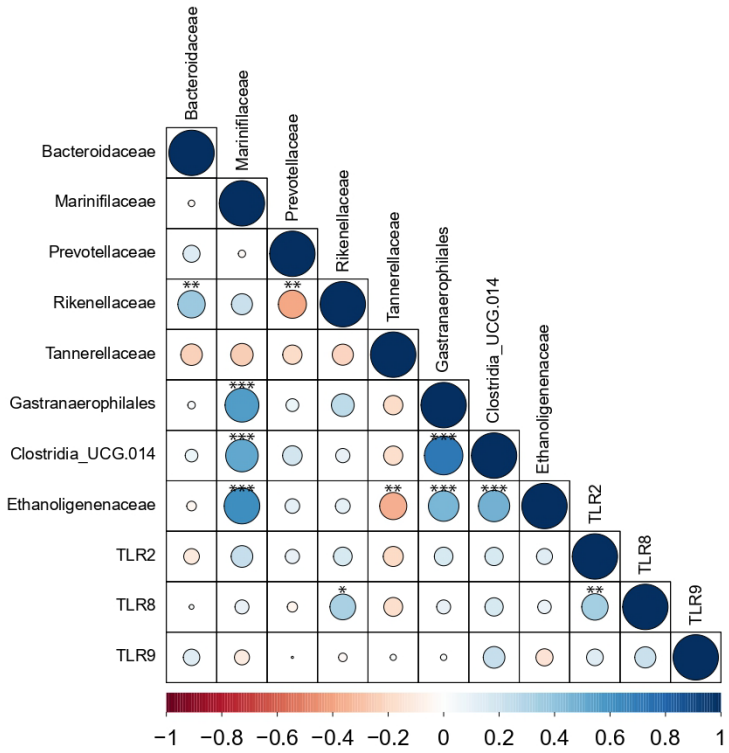

Figure 8. Pearson´s correlation test between the abundance of significant bacterial families (Bacteroidaceae, Marinifilaceae, Prevotellaceae, Rikenellaceae, Tannerellaceae, Gastranaerophilales, Clostridia_UCG.014 and Ethanoligenenaceae) and significant TLRs (2, 8 and 9). Correlation values range from red (negative correlation) to blue (positive correlation) colors and size. *p<0.05; **p<0.01; and ***p<0.001.

Regarding TLR9, this receptor recognizes bacterial CpG DNA. The reduction in TLR9 expression induced by clindamycin observed in our study is in agreement with the effects of other antibiotics like bacitracin and neomycin on the levels of this receptor [42]. However, it has been reported that tetracyclines increase the expression of TLR9 [70]. The activation of TLR9 in epithelial cells suppresses inflammatory signals induced by the activation of other TLRs [71]. In models of experimental colitis, the administration of CpG significantly reduces the proinflammatory cytokine expression of IFN-γ and IL-6, increases anti-inflammatory IL-10 and reduces disease severity [4], being the protective function of TLR9 relatively clear.

TLR8 recognizes viral ssRNAs and has been associated with certain chronic viral disorders [72]. TLR8 signalling has also been reported to increase the generation of TNF-α and IL-1β, both of which are associated with mucosal inflammation in ulcerative colitis [73]. In our study, treatment with clindamycin decreased the expression of TLR8, however in other studies, bacitracin and neomycin increased TLR8 expression [42].

Some authors have studied the effect of Lf on the immune system through TLR modulation. In a rat model of diclofenac-induced enteropathy, the administration of lactoferrin resulted in a further increment of TLR2 expression [74]. In our study, none of the lactoferrins *per se* significantly increased TLR2 expression. However, the administration of sLf along with clindamycin reverted the decrease in the expression of this receptor caused by antibiotic treatment. In this way, another study demonstrated that lactoferrin may suppress Epstein–Barr virus-induced inflammation by interfering with the activation of TLR2 and TLR9 since Lf inhibited the ability of TLR9 to recognize dsDNA thereby reducing proinflammatory IL-8 production [75]. In our results, the animals treated with nLf showed a strong reduction in TLR9 expression and this effect was maintained in the nLf_Clin group. Nevertheless, the level of this receptor in the sLf_Clin group was similar to that of Vehicle, again demonstrating the ability of sLf to reverse the decrease in TLR9 expression caused by clindamycin.

Understanding the relationship between microorganisms residing in the gut and TLRs is interesting for developing compounds capable of maintaining or reestablishing homeostasis in the gut. For this reason, we studied the correlations between certain families of the intestinal microbiota and TLR2, 8 and 9, whose expression was modified by antibiotic treatment. Our results showed a positive correlation between TLR8 and Rikenellaceae family. In line with this finding, a similar study observed that TLR3 and TLR8 were positively associated with the proportion of Rikenellaceae [76]. Little is known about the correlation between the different TLRs. Bösl *et al.* investigated the coactivation of TLR2 and TLR8 in human primary monocytes and they found that TLR2 signal induced an inhibitory loop that reduced TLR8 signal intensity [77]. In contrast to these results, our study showed that TLR8 was positively correlated with TLR2.

In our study, the family Rikenellaceae was positively correlated with the abundance of Bacteroidaceae. Bacteria from these families can reduce intestinal inflammation by inducing anti-inflammatory cytokines such as IL-10 or by suppressing the secretion of inflammatory cytokines [50, 57]. This shared feature could explain the positive correlation shown in this study. In addition, it has been reported that an animal-based diet increases the abundance of *Alistipes* and *Bacteroides*, belonging to Rikenellaceae and Bacteroidaceae families, respectively [78]. It has been shown that

subjects with high levels of *Prevotella* tend to have lower levels of *Bacteroides*, which implies that bacteria of these genera compete for the same niche in the gut [54], however, this negative correlation was not found in our study. In contrast, the family Prevotellaceae was negatively correlated with Rikenellaceae. In this regard, it could be argued that although the negative correlation between Prevotellaceae and Bacteroidaceae is not directly reflected in our results, it is indirectly reflected through the correlations of these two families with Rikenellaceae.

Despite finding differences in the expression of some TLRs and in the abundance of some bacteria between the different groups of mice, we have not found obvious signs of inflammation in the colon of any group of treated mice. In fact, intestinal dysbiosis induced by antibiotics can induce an activation of the immune system but not always inflammation. Mice with intestinal dysbiosis induced by other antibiotics like neomycin and bacitracin showed normal histological structure of colon and no differences in pro-inflammatory cytokines expression [79, 80].

5. Conclusions

Our results show that clindamycin induces alterations in the composition of the intestinal microbiota, reducing bacteria with anti-inflammatory properties such as Bacteriodaceae, Prevotellaceae or Rikenellaceae, and in the expression of TLR2, TLR8 and TLR9 receptors. Both native and iron-saturated lactoferrin are capable of restoring the normal levels of these anti-inflammatory bacteria, but only iron-saturated lactoferrin restored the normal levels of TLR receptor expression. Therefore, our study reinforces the fact that lactoferrin is an ingredient that can be used in functional foods, since it contributes to the maintenance of intestinal health by modulating the intestinal microbiota and host immune system.

Author Contributions

A.Bellés and L. Grasa performed experiments, carried out the data analysis and wrote the manuscript. D. Aguirre-Ramírez, I. Abad and L. Sánchez performed experiments. M.Parras contributed to the data analysis. L. Grasa and L. Sánchez reviewed the manuscript and were responsible for funding acquisition. All authors read and approved the final manuscript.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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