RESEARCH ARTICLE

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Whey and Buttermilk-Based Formulas Modulate Gut Microbiota in Mice with Antibiotic-Induced Dysbiosis

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Scope: Diet is one of the main factors that modifies intestinal microbiota composition. The search for foods that can reverse situations of intestinal dysbiosis such as that induced by antibiotics is of great interest. Buttermilk and whey are the main by-products produced by the dairy industry containing bioactive compounds. The aim of this study is to investigate the ability of whey and buttermilk-based formulas supplemented with lactoferrin and milk fat globule membrane (MFGM) to modulate the effects of clindamycin on mouse intestinal microbiota.

Methods and results: Male C57BL/6 mice are treated with saline (control), clindamycin (Clin), a formula containing whey (F1) or buttermilk (F2), Clin+F1 or Clin+F2, and their fecal microbiota profiles are analyzed by sequencing of 16S rRNA gene using the MinION device. Clin induces alterations in both the composition and metabolic functions of the mice intestinal microbiota. The treatment with F1 or F2 reverses the effects of clindamycin, restoring the levels of Rikenellaceae and Lactobacillaceae families and certain pathways related to short-chain fatty acids production and tetrahydrofolate biosynthesis.

Conclusion: Whey and buttermilk supplemented with lactoferrin and MFGM may be a bioactive formula for functional foods to prevent or restore microbiota alterations induced by antibiotic administration.

1. Introduction

Whey and buttermilk are the main by-products of the dairy industry. Whey is obtained after the coagulation of casein during cheese manufacture. Buttermilk is the liquid phase released during the churning of cream in the butter-making process.[1] About 55 million tons of whey and 2.3 million tons of buttermilk were produced in 2020 in the European Union.[2] In addition to the huge volume produced of these by-products, they exhibit a high biological and chemical oxygen demand due to their levels of organic matter.[3] Despite their polluting potential, both are a valuable source of numerous nutritional, functional, and bioactive compounds, and their use in the agri-food and pharmaceutical industry could contribute to the circular economy strategies.

Whey is composed of about 70% of lactose, 14% whey proteins, 12% minerals, and some vitamins and residual fat.[1,4] Lactose has been proposed to have prebiotic properties in certain situations since it is used as a substrate by beneficial bacteria, such as *Lactobacillus* spp.[5] The enzymatic hydrolysis of lactose, catalyzed by β-galactosidase, released by certain species of the gut microbiota, mainly yields glucose and galactose. In the same reaction, galacto-oligosaccharides are also formed by a trans-galactosylation reaction.[6] These oligosaccharides stimulate the growth and metabolism of the bifidobacteria population. Consequently, lactic acid is produced, and it prevents the growth of some pathogenic bacteria, such as *Salmonella, Escherichia coli (E. coli), Bacteroides,* or *Clostridium*.[5,7,8] Furthermore, whey is a rich source of proteins and bioactive peptides with well-known health

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benefits, such as antimicrobial, antioxidant, antihypertensive, and immunomodulatory properties.[1,9] The major whey proteins are β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins, lactoferrin, and lactoperoxidase, which can be hydrolyzed into bioactive peptides by digestive enzymes or by the action of proteolytic enzymes of microorganisms.[4,7] Previous studies have demonstrated the capacity of whey proteins to modulate the composition of colonic microbiota. These studies showed that whey and whey peptide extract promoted the growth of *Bifidobacterium* spp. and *Lactobacillus* spp. in vivo[10,11] and in vitro models.[7,11]

For many years, buttermilk has been considered an unvaluable by-product of the milk industry. However, over the last decades, buttermilk has attracted a great deal of interest due to its technological functions as emulsifier and its high content of milk fat globule membrane (MFGM). This biological membrane is derived from the mammary gland epithelium and is composed of a complex mixture of proteins, polar and apolar lipids. Polar lipids of MFGM are phospholipids and sphingolipids, and their concentration in buttermilk is five times higher than in whole milk.[12] These bioactive compounds have antitumor, anti-inflammatory and cholesterol-lowering effects, and also may prevent gastrointestinal infections.[1] Indeed, emerging evidence has suggested a protective effect of MFGM against infectious diseases, in part through the modulation of the gut microbiota.[13] The addition of bovine MFGM to formula milk increased the gut microbial richness in rat pups,[14] and decreased the proportions of gastrointestinal pathogens in neonatal piglets.[15]

Given their potential beneficial effects on human well-being, buttermilk, and whey could be used for developing novel functional foods capable of modulating gut microbiota. The intestinal microbiome is the most complex bacterial community in the human body, and it is essential for homeostasis and host health.[16] Gut microbiota composition is continuously exposed to factors that influence it dynamically, such as diet, exercise, lifestyle, and some drugs.[17] Antibiotic treatment has the adverse effect of altering the composition and function of intestinal microbiota and it has been associated with an increasing risk of *Clostridium difficile* colitis, inflammatory bowel disease, and obesity.[18] Moreover, intestinal dysbiosis is linked not only with intestinal disorders but also with extraintestinal diseases, such as metabolic and neurological disorders.[17]

Consequently, it is of great interest to develop new strategies based on dairy by-products to restore the resident microbiota and improve intestinal health. For this reason, the aim of this study was to investigate the effects of whey- and buttermilk-based formulas (F1 and F2, respectively), supplemented with lactoferrin and MFGM, on the composition and function of the intestinal microbiota of mice with antibiotic-induced dysbiosis.

2. Results

2.1. Gut Microbiota Composition

At the phylum level, Firmicutes and Bacteroidota were the predominant phyla representing more than 90% of the gut microbiota (**Figure 1**A). Most of the Bacteroidota sequences belonged to the Bacteroidales order, where Muribaculaceae and Rikenellaceae were the most abundant families. Most of the Firmicutes sequences belonged to the Lachnospirales, Lactobacillales, Oscillospirales, and Erysipelotrichales orders, with Lachnospiraceae, Lactobacillaceae, Erysipelotrichaceae, Oscillospiraceae, and Ruminococcaceae being the most abundant families. All Verrucomicrobiota sequences belonged to the Akkermansiaceae family. Most of the Proteobacteria sequences belonged to the Burkholderiales order. All Desulfobacterota sequences belonged to the Desulfovibrionales order (Figure 1B,C). *Lachnospiraceae_NK4A136_group*, *Lactobacillus*, *Muribaculaceae*, *Lachnoclostridium*, and *Rikenellaceae_RC9_gut_group* were included in the top 5 most abundant genera (Figure 1D).

2.2. Alpha and Beta Diversity

To compare bacterial diversity within groups, we evaluated four α-diversity indexes (**Figure 2**A). Surprisingly, Clinamycin (Clin)+F2 treatment induced an increase in microbiota richness (observed species and Chao1) and diversity (Shannon´s index) compared to Control group. Clin+F1 group induced an increase only in the Chao1 index. No significant differences were observed in the microbiota evenness (Simpson´s index) between the different treatment groups. When we calculated Shannon and Simpson indexes using predicted functions for the microbiota of the different groups, we observed that treatment with Clin decreased significantly both indexes and the treatment with Clin+F2 could reverse these effects, restoring the functional alpha diversity nearly to Control levels (Figure 2B).

In terms of beta diversity, the microbial community of the Control group was statistically different from the communities of the Clin, Clin+F1, and Clin+F2 groups (**Figure 3**A,D,E). In addition, there were also differences between the bacterial communities of the Clin respect to Clin+F1 or Clin+F2 groups (Figure 3F). However, PERMANOVA analysis did not show differences between Control group and F1 or F2 treated mice (Figure 3B,C). When dissimilarities between groups in terms of functional beta diversity were analyzed, similar results were observed (Figure S1, Supporting Information).

Venn diagrams showed that the core bacterial community among the Control, Clin, and Clin+F1 or Clin+F2 groups was composed of 17 or 18 OTUs, respectively (Figure 3G,H). Nevertheless, Control, F1 and F2 groups shared 41 OTUs (Figure 3I), indicating a similar bacterial community structure between these groups.

2.3. Differences in Bacterial Abundance

We analyzed the differences in the abundance of bacteria at the phylum, family, and genera levels in the different groups. Differences in the relative abundance of the different phyla are shown in Figure S2, Supporting Information. A family abundance heatmap was obtained with the 20 families more affected by treatments (Figure S3, Supporting Information). **Figure 4** shows some of the most important families affected by Clin treatment. Compared to the Control group, the relative abundances of Desulfovibrionaceae, Rikenellaceae, Prevotellaceae, Marinifilaceae, and Lactobacillaceae were significantly decreased in the mice treated with Clin (Figure 4C–F,I). The treatment with F1

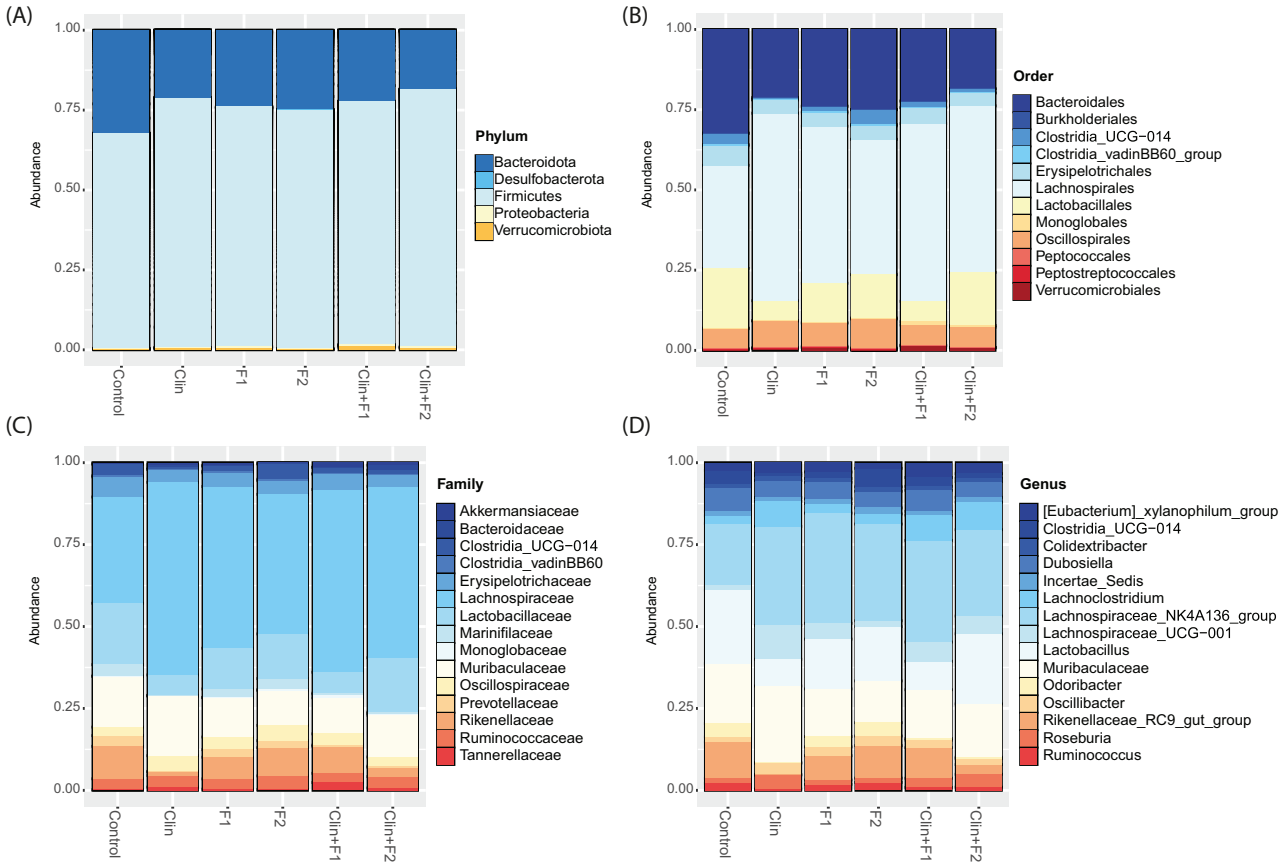


Figure 1. Bacterial relative abundances at phylum (A), order (B), family (C), and genus (D) levels for each treatment (Control, Clin, F1, F2, Clin+F1, and Clin+F2).

and/or F2 along with Clin increased the bacterial levels of Desulfovibrionaceae, Rikenellaceae, and Lactobacillaceae families to similar levels of Control group (Figure 4C,D,I). Nevertheless, the relative abundances of Akkermansiaceae, Tannerellaceae, Lachnospiraceae, and Oscillospiraceae were increased in Clin group, and this effect was maintained in mice treated with Clin+F1 and Clin+F2, except for Oscillospiraceae family (Figure 4A,B,G,H).

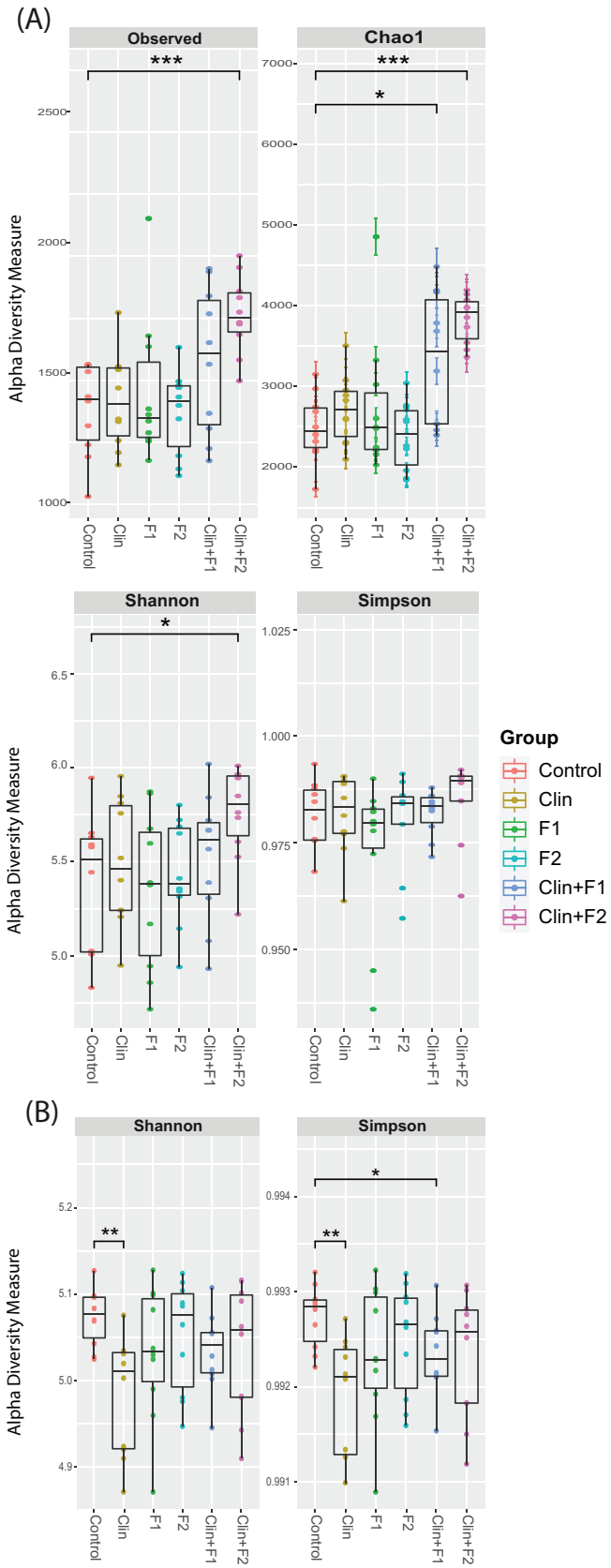
At the genus level, Clin group harbored higher proportions of *Parabacteroides* (family Tannerellaceae) and *Lachnospiraceae_NK4A136_group* (family Lachnospiraceae) (**Figure 5**A,B). In animals treated with F1 or F2 together with the antibiotic, the increase in these bacterial genera was also maintained (Figure 5A,B). However, there were significant declines in the abundance of *Rikenellaceae_RC9_gut_group* (family Rikenellaceae), *Lactobacillus* (family Lactobacillaceae), *Prevotellaceae_UCG-001* (family Prevotellaceae), and *Odoribacter* (family Marinifilaceae) after Clin administration (Figure 5C–F). In this case, the treatment with F1 or F2 along with Clin was able to restore partially the levels of *Rikenellaceae_RC9_gut_group* and *Lactobacillus* genera (Figure 5C,D).

2.4. Differences in Functional Pathways

The functional metabolic profiling resulting from PICRUSt2 genome prediction produced 336 metabolic pathways. We assessed whether there were differences in the functional capacity of the microbiota among the different treatments. We identified 109 pathways that were differentially abundant ($p < 0.05$) between Control and Clin group, whereas in Clin+F1 and Clin+F2, only 21 and 67 pathways, respectively, were differentially abundant compared to Control. No differences were observed with F1 and F2 treatments. Then, we selected the nine metabolic pathways most affected by antibiotic treatment and analyzed their expression among the different groups (**Figure 6**). Clin+F1 reverted the decline induced by Clin treatment in FOLSYN−PWY (superpathway of tetrahydrofolate biosynthesis and salvage), P163−PWY (L-lysine fermentation to acetate and butyrate), PWY−6612 (superpathway of tetrahydrofolate biosynthesis), and PWY−7090 pathways (UDP-2,3-diacetamido-2,3-dideoxy-α-D-mannuronate biosynthesis) (Figure 6A,D,H,I). In Clin+F2 group, HEXITOLDEGSUPER−PWY (superpathway of hexitol degradation), P441−PWY (superpathway of N-acetylneuraminate degradation), and PWY−7090 (UDP-2,3-diacetamido-2,3-dideoxy-α-D-mannuronate biosynthesis) pathways had similar expression to Control animals (Figure 6C,F,I).

3. Discussion

In previous research, we demonstrated the protective effect of native lactoferrin on intestinal dysbiosis induced by Clin.[19] Other authors have tested the effects of MFGM supplementation on



intestinal development and the microbiome as well as its potential to protect against *C. difficile*-induced colitis.[14] Moreover, the potential of whey proteins in the modulation of gut microbiota has also been studied.[11,25] These findings provide evidence of the individual effects of these milk bioactive components on microbiota, however, there are still gaps regarding the possible application of functional foods made from a combination of these components. Therefore, in this study we evaluated the potential of two formulas, containing a mixture of lactoferrin, MFGM, and whey or buttermilk, to reverse Clin-induced effects on intestinal microbiota.

Regarding the bacterial composition of the samples, most of the sequences were assigned to the Firmicutes and Bacteroidota phyla. The mammalian gut is colonized by hundreds of different bacterial species, most of which belong to the phyla Firmicutes and Bacteroidetes.[26] In terms of microbial alpha diversity, Clin treatment did not modify any of the alpha diversity estimators, indicating that a single dose of Clin does not decrease microbial diversity, which is consistent with our previous study.[19] The two species richness estimators (Observed OTUs and Chao 1) were higher in the Clin+F2 treatment. Richness estimators such as Chao1 mainly depend on the number of rare OTUs.[27] Nevertheless, diversity depends not only on richness but also on evenness. This would indicate that in the group treated with F2 along with the antibiotic there is an increase in richness due to rare species. Shannon diversity index is an estimator of species richness and evenness but has more weight on species richness. Simpson´s index has more weight on species evenness.[28] This could explain why Shannon index was also significantly higher in the Clin+F2 group, but there was no difference with Simpson´s index. This result would indicate that in the group treated with F2 along with the antibiotic diversity was increased only in terms of species richness. The alpha diversity of the intestinal microbiota from mice treated with Clin and lactoferrin was not modified,[19] indicating that buttermilk and/or MFGM could be responsible for this effect.

The diversity of functions performed by organisms within ecosystems is even of greater interest than their identity.[29] In our study, although taxonomic diversity was similar in the Control and Clin groups, when we analyzed functional traits between groups, we observed lower functional diversity in the microbiota of mice treated with Clin compared to Control animals. Nevertheless, Shannon index was not significantly modified in the Clin+F1 and Clin+F2 groups respect to Control, indicating that F1 and F2 would preserve some microbial pathways.

The beta diversity analysis showed that animals treated with Clin clustered together and were clearly separated from the Control group, which was clustered with the F1 and F2 treated animals. Thus, this suggests that Clin treatment modifies the structure of the intestinal microbiota community and its functionality.

Figure 2. A) Alpha-diversity indexes (Observed species, Chao1, Shannon and Simpson) across the different treatment groups using taxonomic data. B) Microbial functional diversity (Shannon and Simpson) across the different treatment groups. Values are presented as median ($n = 10$). Significance was assessed using Wilcoxon test and denoted as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

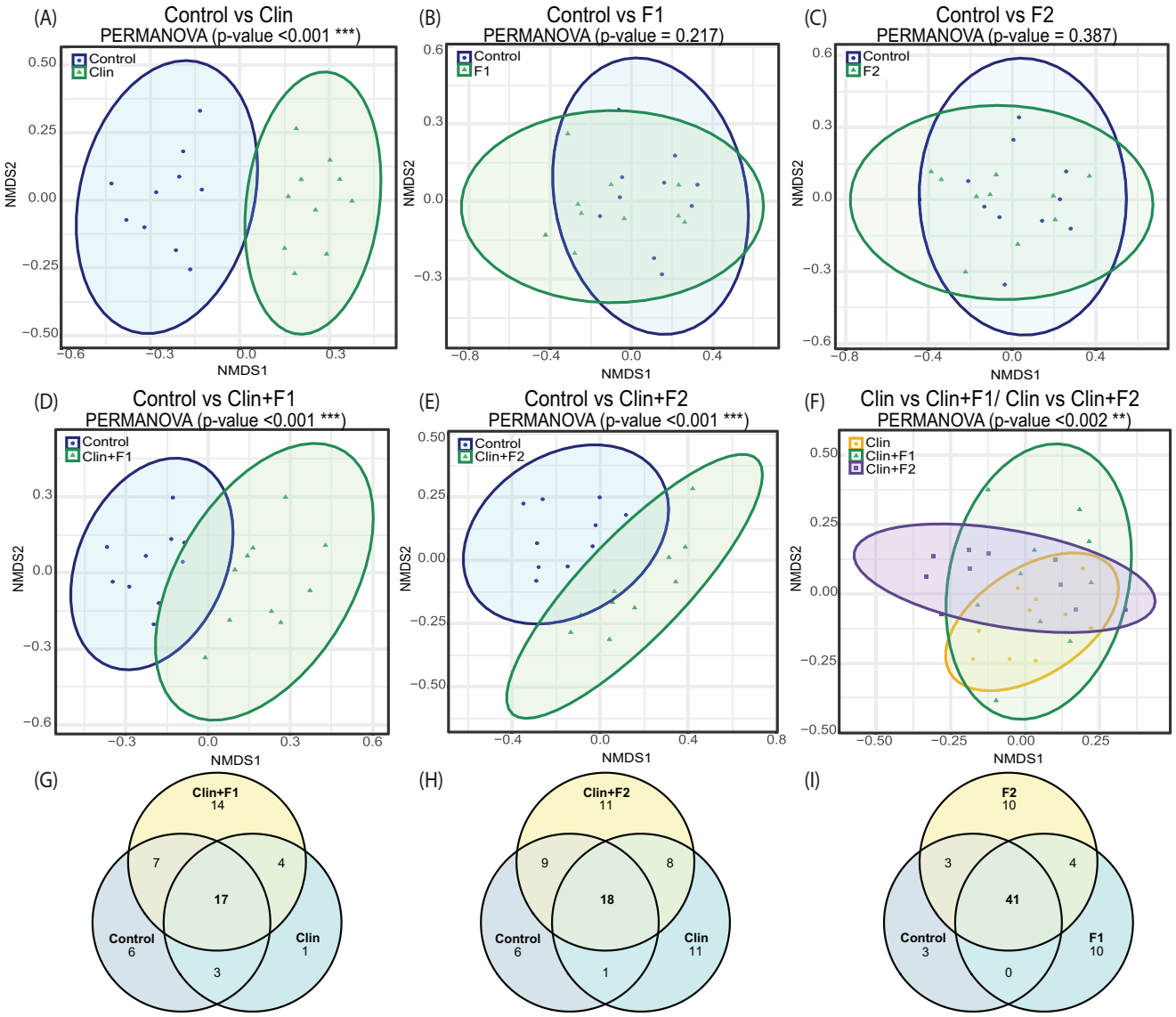


Figure 3. Beta-diversity represented by NMDS showing the Bray–Curtis distances between the microbial populations of the Control group versus the different treatments (A–E) and Clin versus Clin+F1 versus Clin+F2 (F). Significance between each pair of groups was assessed using a PERMANOVA test and denoted as follows: $^{**}p < 0.01$, $^{***}p < 0.001$. Venn diagrams indicating the number of shared and unique OTUs among the groups: Control, Clin and Clin+F1 (G); Control, Clin and Clin+F2 (H); and Control, F1 and F2 (I).

This result agrees with our previous study with this antibiotic.[19] Regarding the treatment with F1 or F2 along with antibiotic, we observed significant differences between these groups with respect to the Control group. Nevertheless, core microbiota analysis showed that Clin+F1 and Clin+F2 groups shared more species with Control than with the Clin group, indicating that dairy formula treatments could mitigate clindamycin-induced changes in certain taxa of the intestinal microbiota. Moreover, analysis of predicted functional pathways revealed that the number of altered pathways in Clin+F1 and Clin+F2 groups was much lower than in animals receiving only Clin.

A deeper exploration into more specific taxonomic groups (families and genera) showed that Clin treatment strongly decreased the abundance of bacteria belonging to Bacteroidota phylum (Marinifilaceae, Prevotellaceae, and Rikenellaceae families). This is consistent with the depletion of Bacteroidota community observed by other authors after Clin administration.[19,30,31]

The treatment with F1 along with Clin increased the levels of Rikenellaceae family and *Rikenellaceae_RC9_gut_group* genus similarly to those of the Control group. Members of the family Rikenellaceae are hydrogen-producing bacteria. In the inflammation process, H_2 mediates the suppression of pro-inflammatory cytokines, especially IL-1β, TNF-α, and IL-6, in inflamed tissues.[32] It has been reported that endogenous hydrogen reduces oxidative stress and ameliorates the symptoms of inflammatory bowel disease, which would improve the quality of life of patients.[33] Therefore, F1 administration could mitigate the decline in Rikenellaceae family observed after Clin

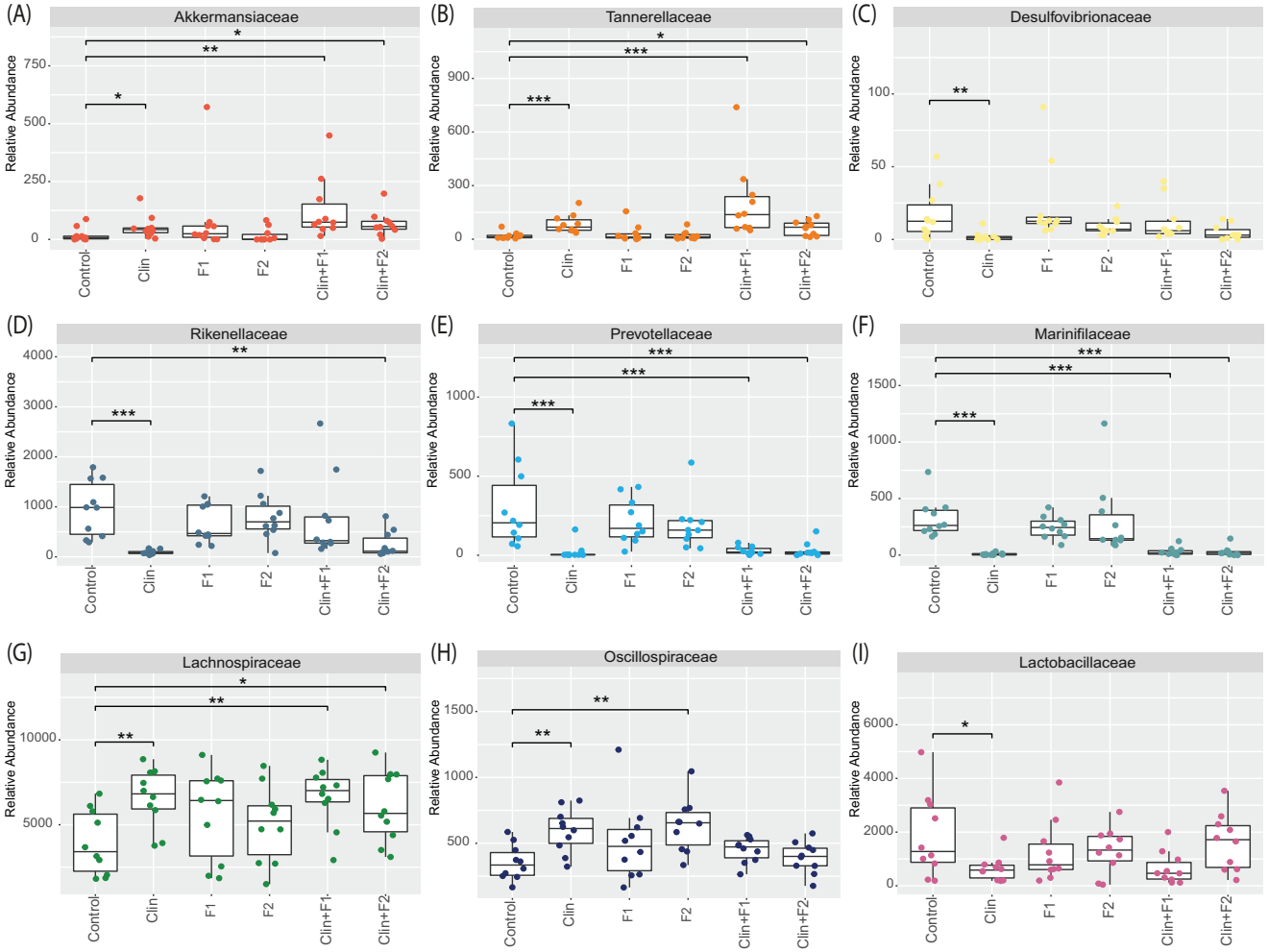


Figure 4. Relative abundances of selected families for each treatment (Control, Clin, F1, F2, Clin+F1, and Clin+F2). Values are presented as median (n = 10). Significance was assessed using Wilcoxon test and denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

treatment and modulate positively the levels of this beneficial bacteria. According to previous results, the effects of F1 and lactoferrin alone on this bacteria family were similar,[19] indicating that whey or MFGM do not potentiate the effect of lactoferrin.

In contrast, Clin treatment evoked an increase in the family Tannerellaceae, belonging to Bacteroidota phylum. This result is in agreement with our previous study on Clin-induced dysbiosis.[19] The family Tannerellaceae includes *Parabacteroides* and *Tannerella* genera. The *Parabacteroides* genus is associated with T-cell differentiation by enhancing and maintaining the IL-10 producing Treg cells. Some *Parabacteroides* species have the ability to reduce intestinal inflammation by inducing the antiinflammatory cytokine IL-10 and suppressing the secretion of inflammatory cytokine IL-17, IL-6, and IFN-γ.[34,35] Both F1 and F2 administration maintained the high levels of the bacteria of the Tannerellaceae family and genus *Parabacteroides* induced by Clin treatment, suggesting that these formulas maintain the positive effects of Clin on the levels of these anti-inflammatory bacteria. According to previous results, the effects of F1 and F2 and lactoferrin alone on this bacteria family were similar,[19] indicating that whey, buttermilk, or MFGM do not potentiate the effect of lactoferrin.

The impact on families within the phylum Firmicutes was heterogeneous. Clin appeared to inhibit some families (Lactobacillaceae) while others proliferated (Lachnospiraceae, and Oscillospiraceae). In this case, both F1 and F2 treatments succeeded in modulating the changes induced by Clin on Lactobacillaceae family. In line with our findings, in other studies, levels of species belonging to *Lactobacillus* genera significantly decreased in antibiotic-treated animals compared with control animals.[36,37]

Lactobacillaceae is a diverse family of lactic acid bacteria found in the gut microbiota of humans and many animals. These bacteria have beneficial effects on intestinal health, such as modulation of the immune system and protection against pathogens, which is why many species are frequently used as probiotics.[8] The *Lactobacillus* genus enhances intestinal barrier function by increasing mucus production, stimulating the release of antimicrobial peptides and the production of secretory immunoglobulin A, increasing tight junction integrity of intestinal epithelium, and providing competitive resistance against pathogens.[38]

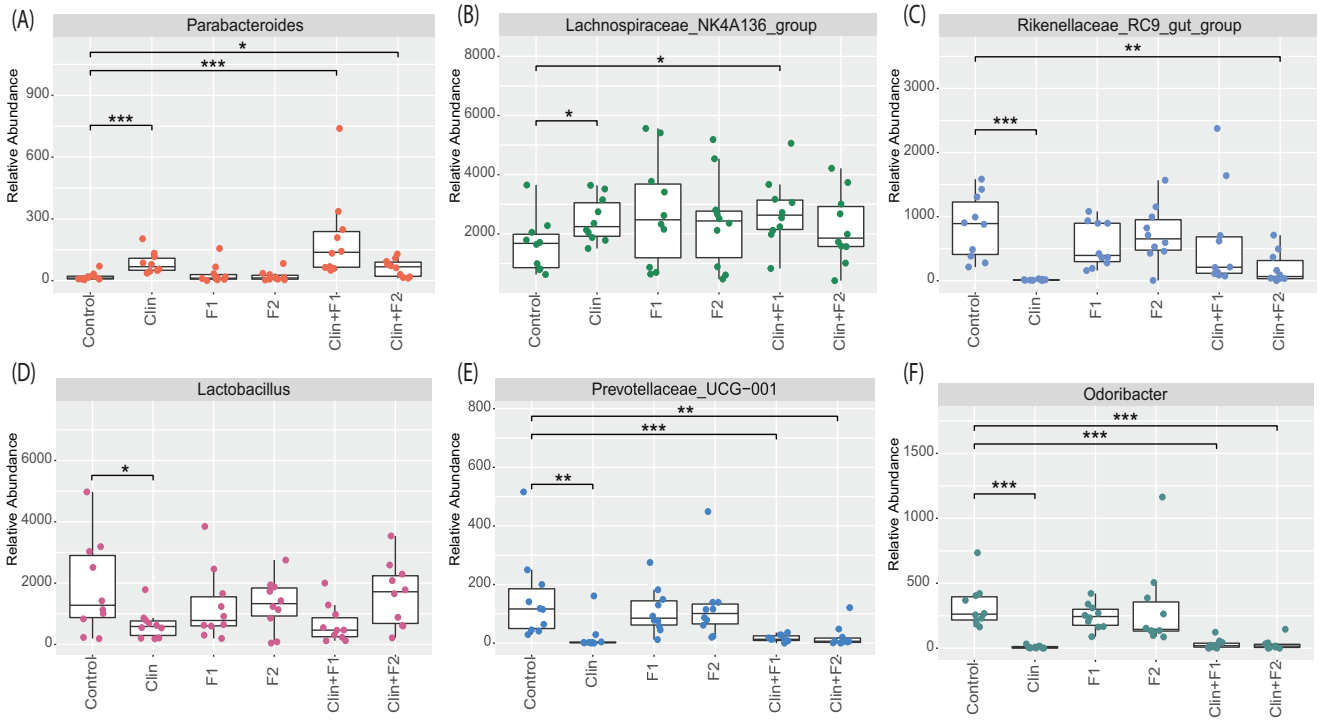


Figure 5. Relative abundances of selected genera for each treatment (Control, Clin, F1, F2, Clin+F1, and Clin+F2). Values are presented as median (n = 10). Significance was assessed using Wilcoxon test and denoted as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Lactobacillus strains play an immunological role within the GI tract of the host, as they can interact with both the innate and adaptive immune response. For example, in an in vitro study of chicken splenic and cecal tonsil cells, *L. acidophilus* and *L. salivarius* induced Th1 and cytokine anti-inflammatory responses, respectively.[39] Inflammatory bowel disease (IBD) is an example of an intestinal inflammatory disease that may be modulated by different *Lactobacillus* species. Mice administered *L. reuteri* strains and exposed to DSS showed reduced severity of colitis.[40] Therefore, the restoration of the levels of this important genus by the administration of F1 or F2 could have beneficial effects on intestinal pathologies involving dysbiosis. It has been demonstrated that the inclusion of whey protein in the diet significantly increases fecal counts of *Bifidobacterium* spp. and *Lactobacillus* spp.[11,41] Nevertheless, the effect we observed was greater with the F2 treatment than with F1. This could be attributed to the high MFGM content of F2, as it is based on buttermilk that is very rich in this fraction. Furthermore, it has been reported that some MFGM glycoproteins are able to survive gastric digestion.[13] Therefore, this membrane may confer a prebiotic effect, providing an energy source to support the growth of colonic bacteria.[42]

Regarding Lachnospiraceae family, one of the major taxonomic groups of the human gut microbiota, it has been associated with the maintenance of gut health. In our study, the levels of Lachnospiraceae were increased by Clin treatment and this positive effect was maintained in Clin+F1 and Clin+F2 groups. Members of this family have been linked, on the one hand, to obesity and, on the other hand, to protection against colon cancer in humans. This positive effect is due to the relationship of many species with the production of butyric acid, a SCFA that is associated with the control of gut inflammatory processes and is important for the intestinal epithelial barrier maintenance.[43]

Clin treatment induced an increase in the family Akkermansiaceae, some mucin-degrading bacteria that reside in the intestinal mucosal layer and have been associated with a healthy gut mucosa.[44] *Akkermansia*, as a potential probiotic agent, has been reported to reduce mucosal inflammation via the improvement of the microbial community and gut barrier function in DSS-induced colitis mice.[45] In Clin+F1 and Clin+F2 groups the abundance of the Akkermansiaceae family also increased compared to the Control, indicating that these milk formulas maintained this positive effect induced by Clin administration.

Similarly to previous results of other authors,[46] Clin treated mice in our study showed a decrease in Desulfovibrionaceae family. Several species of the Desulfovibrionaceae family can generate hydrogen sulfide (H_2S). In an innovative study on a synthetic microbiota community, H_2S was found to inhibit butyrate production, including the members *Faecalibacterium prausnitzii* and *Roseburia intestinalis*.[47] Moreover, H_2S produced by the Desulfovibrionaceae family could reduce the disulfide bonds of the intestinal mucosal layer and cause epithelium exposure to bacteria and toxins, which could lead to inflammation.[48] In our study, F1 and F2 formulas partially increased the levels of this family, which had been strongly reduced by Clin treatment.

Taken together, our results indicate that F1 and/or F2 ingestion normalizes Clin-induced decline in Rikenellaceae and Lactobacillaceae, two important families with well-known effects on

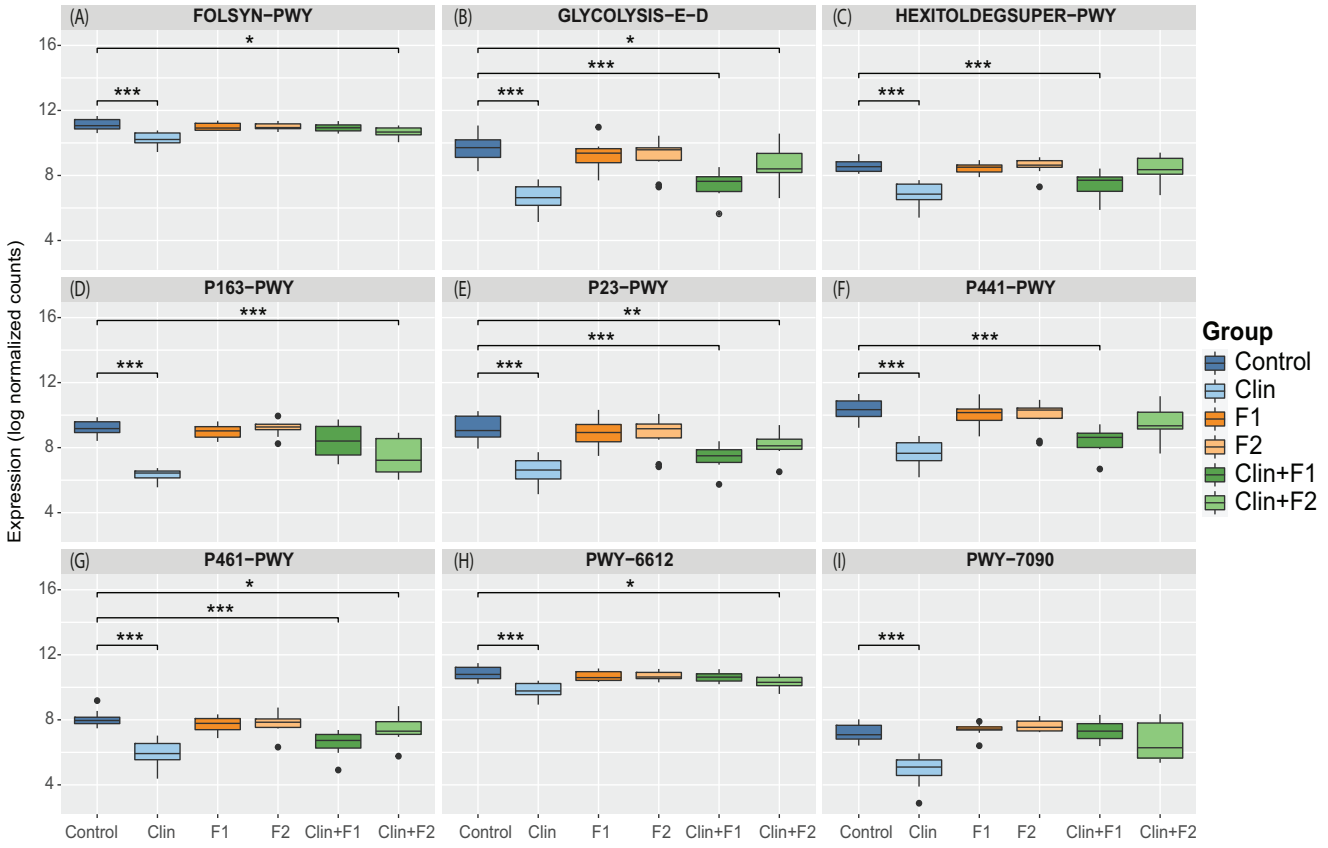


Figure 6. MetaCyc functional pathways identified for each treatment (Control, Clin, F1, F2, Clin+F1, and Clin+F2). Values of each functional gene were log2 transformed. Tests of significance of the gene distribution between groups were performed using Wilcoxon test and denoted as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

intestinal health. Furthermore, we have also observed that Clin increases the levels of the Tannerellaceae, Lachnospiraceae, and Akkermansiaceae families, which have anti-inflammatory properties and are important for gut health, and this positive effect is not modified by either F1 or F2. However, both milk formulas reverse the decline produced by Clin in the Desulfovibrionaceae family, which has harmful effects on host health.

Regarding the microbiota functional profile, Clin treatment decreased the P163-PWY pathway, which is related to L-lysine fermentation to acetate and butyrate. Several genera of bacteria have been shown to ferment L-lysine as a sole source of carbon and nitrogen, producing butyrate and acetate.[49] In addition, two pathways related to cofactor and vitamin biosynthesis, specifically, related to tetrahydrofolate (vitamin B9) biosynthesis, were reduced in antibiotic-treated mice. Folates are essential cofactors that facilitate the transfer of one-carbon units from donor molecules into important biosynthetic pathways leading to methionine, purine, and pyrimidine biosynthesis. This pathway for the de novo biosynthesis of folates is found in bacteria, fungi, and plants, whereas vertebrates are absolutely dependent on nutritional sources, making folate a vitamin.[50] The treatment with Clin+F1 was able to restore the levels of these functional pathways and in this context, F1 could be a good ingredient to improve the intestinal health.

4. Conclusions

The present study shows that a single dose of Clin induces alterations in both the composition and metabolic functions of the mice intestinal microbiota. The treatment with whey or buttermilk supplemented with native bovine lactoferrin and MFGM modulates the microbiota composition and the functional pathways adversely affected by antibiotic administration. Although this study does not allow us to attribute specific outcomes to the individual bioactive compounds, these results demonstrate that whey-based formula restored the normal levels of Rikenellaceae family and buttermilk-based formula increased *Lactobacillus* genus. From these results, we suggest that this combination of bioactive ingredients can be used in functional foods to prevent microbiota alterations caused by antibiotic-induced dysbiosis.

5. Experimental Section

Buttermilk and Whey-Based Formulas Preparation: Two formulas were prepared on the basis of whey and buttermilk and enriched with native bovine lactoferrin (nLF) used in the previous research.[19] This nLF (iron saturation below 10%) was kindly provided by Tatua Nutritionals Company (Morrinsville, New Zealand). To obtain whey and buttermilk, raw bovine milk was provided by Villacorona (El Burgo de Ebro, Spain), and

processed at the Food Science and Technology Pilot Plant of the University of Zaragoza, as detailed previously.[20] Formula 1 (F1) was prepared with a whey base, supplemented with nLF and MFGM. For its preparation, freeze-dried whey was rehydrated, taking into account its dry matter (0.068 g mL^{-1}). Once dissolved in 50 mL of miliQ water, nLF (175 mg mL^{-1}) was added. This process was carried out under constant agitation at 4 °C to ensure its correct dissolution. Finally, the MFGM precipitate obtained by centrifugation at 40 000 × g for 30 min of 50 mL of buttermilk was added. To dissolve and homogenize the MFGM in the formula, an ultra-turrax was used. Formula 2 (F2) was prepared with a base of 50 mL of buttermilk, obtained from cream. nLF (175 mg mL^{-1}) was added to buttermilk and slowly dissolved under stirring at 4 °C. Similarly to F1, MFGM obtained from 50 mL of buttermilk by centrifugation was added and homogenized.

Animal Treatments: All procedures were conducted under Project Licence PI40/17 and approved by the Ethics Committee for Animal Experiments of the University of Zaragoza. Sixty male C57BL/6 mice (8–12 weeks old, Janvier Labs, Le Genest-Saint-Isle, France) were randomly divided into six groups ($n = 10$ per group): Control, clindamycin (Clin), F1, F2, Clin+F1, and Clin+F2. Control group received saline by gastric gavage for 10 days. Mice treated with Clin were gavaged for 10 days with saline, and on day 4 received a single IP injection of 200 µg of Clin (Normon Laboratories, Madrid, Spain) diluted in 200 µL of saline. Mice from the groups F1 and F2 were treated for 10 days by gastric gavage with 0.2 mL F1, or F2, respectively. Mice from Clin+F1 and Clin+F2 groups were gavaged for 10 days with 0.2 mL F1 or F2, and on day 4 received an IP injection of 200 µg of Clin.

Sample Collection and Bacterial DNA Extraction: At the end of the treatments, stool samples were collected in a sterile recipient and stored at −80 °C until processed. Bacterial DNA was extracted from fecal samples using the NZY Soil gDNA Isolation kit (NZYTech, Lisboa, Portugal). Two mouse fecal pellets were mixed in NZYSpin Soil Bead Tubes and processed by using the Precellys 24 homogenizer (Bertin Instruments, France). Finally, DNA was eluted in 50 µL elution NSE buffer. DNA concentrations were measured using a Qubit 4.0 fluorometer (Invitrogen, Life Technologies, Madrid, Spain).

Sequencing of 16S rRNA Gene Amplicons: Extracted DNA from each sample was used as a template for PCR amplification of 16S rRNA gene. Full-length 16S rRNA gene (V1–V9) was amplified using 27F and 1492R primers contained in the 16S Barcoding kit SQK-RAB204 (Oxford Nanopore Technologies, Oxford, UK). The reaction volume was 50 µL containing 10 ng of template DNA, 25 µL of LongAmp Taq 2X master mix (New England Biolabs, Ipswich, MA, USA), and 1 µL of 16S barcode primer, at 10 µM. Amplification was performed with the following PCR conditions: initial denaturation at 95 °C for 1 min, 27 cycles at 95 °C for 20 s, 55 °C for 30 s, and 65 °C for 2 min, followed by a final extension at 65 °C for 5 min. Amplified DNA was purified using the Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA), and the amount of DNA eluted with 10 µL of buffer solution (10 mM Tris–HCl pH 8.0, with 50 mM NaCl) was quantified by Qubit 4.0 fluorometer.

The sequencing libraries were prepared using the rapid sequencing amplicons protocol with SQK-RAB204 kit (Oxford Nanopore Technologies). Twelve barcoded libraries were pooled and incubated with 1 µL of Rapid Adapter at RT for 5 min. The libraries were loaded onto the flow cells and sequenced on the MinION (Oxford Nanopore Technologies). MinKNOW software (version 4.1.22) was used for data acquisition. Raw reads with enough quality were subjected to further analysis.

Bioinformatic Analysis: Processing and analysis of reads were carried out using the MetONTIIME pipeline[21] and QIIME2 v.2022.2. Base-calling of the FAST5 files was performed by Guppy_basecaller v.6.1.2 (Oxford Nanopore Technologies). Reads were demultiplexed and adapters and primers were trimmed by Guppy_barcoder v.6.1.2 (Oxford Nanopore Technologies). Sequences were filtered based on read quality and length using NanoFilt v2.7.1.[22] Before clustering, samples were normalized to the minimum number of reads. The reads were dereplicated and clustered into operational taxonomic units (OTUs) at 70% identity. Consensus sequences were assigned to taxonomy using SILVA138_99 as a reference database and the classifier VSEARCH of QIIME 2 plugin.

Output data were imported to R software v.4.2.1, using qiime2R[23] to obtain a phyloseq object. This object was then filtered to remove the least representative OTUs. The differential abundance of bacteria between each pair of groups was compared using the Wilcoxon rank test ($p < 0.05$). Heatmap was obtained using DESeq2 and pheatmap R packages. Venn diagrams were performed to illustrate the treatment effects on the core microbial community, which was defined as the number of OTUs shared among 75% of individuals in each group, using the microbiota and the eulerr R packages.

Alpha and beta diversity were determined using the phyloseq and vegan R packages. Alpha diversity was estimated by richness (observed OTUs and Chao1 index), together with the Shannon and Simpson diversity indexes. Changes in these diversity indexes among the different groups were analyzed using the Wilcoxon test ($p < 0.05$). Bacterial community dissimilarities between treatments were calculated using the Bray–Curtis distance matrix and displayed using the non-metric multidimensional scaling (NMDS) ordination method. To test significant differences between each pair of groups, permutational multivariate analysis of variance (PERMANOVA) tests were calculated (999 permutations, $p < 0.05$).

The QIIME2 plugin for PICRUSt2 was used to predict the functional profile of microbial communities from their 16S amplicon sequences. For this purpose, the OTUs were placed into a reference tree (NSTI cut-off value of 2) using SEPP[24] as a placement tool and the maximum Parsimony (mp) prediction method. As a result, EC, KO, and MetaCyc pathway predictions were obtained to describe the functionality of the microbiota. To compare the functional profiles of the different groups, alpha and beta diversity metrics were calculated and the DESeq2 R package allowed us to identify significantly different functions between sample groups.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

A.B.: performed the experiments, carried out the data collection and analysis, and wrote the first draft of the manuscript. I.A.: performed the experiments. L.S.: performed the experiments and was responsible for funding acquisition. L.G.: performed the experiments, supervision, reviewed the manuscript, and was responsible for funding acquisition. All authors read and approved the final manuscript.

Data Availability Statement

The data of the sequences are available in the NCBI Sequence Read Archive (BioProject ID PRJNA935668).

Keywords

buttermilk, clindamycin, gut microbiota, lactoferrin, whey

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