RESEARCH ARTICLE

Lactococcus lactis D4 Has Potential Effect to Alleviate Inflammation and Reverse Dysbiosis in Colitis Rat Model

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Abstract

BACKGROUND: Inflammatory bowel disease (IBD) is a prevalent chronic inflammatory disorder of the gastrointestinal tract that may lead to colorectal cancer if untreated. Several medications for IBD have adverse side effects. Thus, there are high interest for searching alternative treatment to treat IBD. *Lactococcus lactis* D4 isolated from *dadih*, a traditional fermented buffalo milk product, was investigated for the effect on gut inflammation and microbial composition in the colitis rat model.

METHODS: Eighteen male Sprague-Dawley rats were divided into three groups; control rats, colitis-induced rats, and colitis-induced rats treated with *L. lactis* D4 isolate. The control group received water, while the colitis-induced rats were given azoxymethane (AOM) and dextran sodium sulphate (DSS). Rats' feces were collected for the analysis of gut microbiota diversity by next-generation sequencing and for the measurement of transforming growth factor (TGF)-β, nuclear factor kappa-B (NF-κB), interleukin (IL)-6, and tumor necrosis factor (TNF)-α colonic expression using reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

RESULTS: *L. lactis* D4 administration was able to reduce inflammation in colitis by decreasing IL-6 expression (0.87 *vs.* 0.73), while expression of TGF-β, NF-κB, and TNF-α were increased compared to the model group, suggesting a complex immunomodulatory effect. Gut microbiota sequencing revealed a similarity between the control and treatment groups, suggesting *L. lactis* D4 has great potential to ameliorate gut microbiota dysbiosis in colitis rats.

CONCLUSION: *L. lactis* D4 has a beneficial effect in decreasing pro-inflammatory cytokines and is able to reserve dysbiosis in colitis rat model. Thus, *L. lactis* D4 might be used as a therapeutic agent for IBD.

KEYWORDS: colitis, gut microbiota, inflammation, inflammatory bowel disease, *Lactococcus lactis* D4, probiotic, proinflammatory cytokines

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Introduction

Inflammatory bowel diseases (IBD) are chronic and non-specific inflammatory disorder that are characterized by recurrent and intermittent exacerbations and remissions of clinical symptoms. These symptoms can last for several months to years and can even develop into colorectal cancer if left untreated.(1) Crohn's disease and ulcerative colitis are the two primary types of IBD. Globally, the incidence of IBD was estimated to be approximately 6.8 million cases in 2017.(1-3) Treatment of IBD often involves the use of medications such as corticosteroids, immunosuppressants, and biological agents, which can have significant side

effects including increased risk of infections, liver damage, and potential for malignancy. These treatment challenges necessitate the exploration of safer therapeutic alternatives.(4-5)

Colitis, one of the main types of IBD, shows a particularly high incidence, which significant healthcare challenges, especially in regions like Indonesia where the prevalence ranges from 5.4% to 26.5%. The peak incidence of colitis typically occurs between the ages of 15 and 35 years, although it can manifest in any decade of life. The variability in incidence rates and the broad age range at which colitis can occur underline the need for a tailored approach to management and treatment within the Indonesian healthcare system. The high incidence rates suggest a pressing need for enhanced public health strategies, increased awareness, and improved treatment protocols to effectively manage colitis and prevent its severe complications, including colorectal cancer.(1-3)

The pathogenesis of colitis involves imbalance in the gut microbiota, termed dysbiosis. This imbalance is characterized by a decrease in beneficial bacteria such as *Bifidobacterium* sp., and an increase in potentially harmful bacteria like Proteobacteria and *Fusobacterium* sp. The resultant microbial imbalance contributes to oxidative stress and metabolic disturbances, adversely affecting nutrient transport and absorption, and increasing the risk of colitis and subsequent colorectal cancer.(7-9)

The beneficial effects of probiotics have been known for over a century. By modifying the microbiota, probiotics can both prevent and treat digestive disorders such as colitis. Probiotics help reduce the risk of colitis through various mechanisms such as reducing microbial genotoxicity, altering metabolites produced by the microbiota, competing with harmful bacteria, improving intestinal barrier function, enhancing host innate immune responses, and adjusting cell proliferation and apoptotic processes.(10-12)

A traditional fermented milk from Sumatera Barat, Indonesia, which locally known as *dadih*, is well known to contains probiotics. This fermented milk is made from buffalo milk and naturally fermented with microorganisms. One of the probiotic bacteria found in *dadih* is *Lactococcus lactis* D4. This species of probiotics has been researched before and has been found to have many benefits for the digestive system, such as suppressing various cytokines that play a role in the inflammatory process. *Lactobacillus plantarum* strain CLP-0611 were reported could downregulate the expression of interleukin (IL)-6 and IL-1β. However, there is currently no research on the benefits of *L. lactis* strain D4 on the composition of intestinal microbiota, inflammatory processes, and the incidence of colorectal cancer due to colitis. Therefore, this study was conducted to investigate the effect of *L. lactis* D4 administration on the inflammatory response and regulation of gut microbiota in colitis-induced rats.

Methods

Animal Model and Ethical Approval

Eighteen male Sprague-Dawley rats (INA-Lab, Padang, Indonesia), aged 24-28 weeks old and weighed 170-220 g, were housed and acclimatized in a 30x20x10 cm cage in a controlled environment (25°C, 12-hours light/dark cycle) for 1 week before the experiment. The cage was not exposed to direct light, and the rats were given food and water *ad libitum*. The protocol of this study was approved by the Ethics Committee of Faculty of Medicine, Universitas Andalas (No. 79/UN.16.2/KEP-FK/2023).

Purification of *L. lactis* D4 Culture

L. lactis D4 used in this study was found in *dadih* and was produced at the Faculty of Animal Husbandry, Universitas Andalas.(14) The streak quadrant method was used on MRS agar media to culture *L. lactis* D4. The cup was wrapped to minimize contamination and incubated at 30°C for 48 hours. Once the bacteria had grown, a suspension of material containing bacteria was taken from the agar media using an ose needle and dipped in 10 mL of MRS Broth media. It was then incubated for 24 hours at 30°C. After the incubation process, the resulting *L. lactis* D4 was put into a microtube with 1 mL of volume and centrifuged at 10,000 rpm for 10 minutes at 4°C. This separated the supernatant and pellet, with the supernatant being discarded and 200 µL NaCl added to the *L. lactis* D4 pellet. The isolate was then put into a microtube with a volume of 0.5 mL according to the dose given to the experimental animals, which was $1x10^9$ CFU/mL.

Experimental Design

The study was designed using a post test-only randomized control group design. A total of 18 rats were randomly divided into three groups: control group, colitis-induced group, and colitis-induced treated with *L. lactis* D4 isolate group. The determination of rats number in each group (n=6) was based on WHO guidelines.(13) After acclimatization, 6 rats were randomly selected for the control group, while the remaining 12 rats were used as colitis models by inducing 10 mg/kgBW of carcinogen azoxymethane (AOM) (Sigma-

Aldrich, St. Louis, MO, USA) intraperitoneally, followed by one cycle of dextran sodium sulphate (DSS) (Sigma-Aldrich). As much as 0.5% of DSS was mixed in drinking water for five days. The determination of colitis in rats was based on the results of previous preliminary research. The treatment group was given *L. lactis* D4 isolate, which was administered per rectal every day for two weeks, starting from week 3 at a dose of 10^9 CFU/mL. After two weeks, the feces of each group were collected in amounts of 5 mg. At week 13, the rats were euthanized by cervical dislocation, and colon tissue was collected through surgery to identify colorectal cancer (Figure 1).

Histopathology Examination

Colon tissue was processed into paraffin blocks, cut at 4 µm thick and stained with hematoxylin-eosin (HE) for confirmation of colitis or colorectal cancer examined by a pathologist.

Next Sequencing Generation Analysis of Gut Microbiota

The DNA of feces samples was extracted using a Magnetic Soil and Stool DNA Kit (Tian Gen, Beijing, China). Methods of sample quality control refer to the quality control report. Quality control progress was based on data split, sequence assembly, data filtration, and chimera removal.

Specific primers CCTAYGGGRBGCASCAG and GGACTACNNGGGTATCTAAT were used to amplify 16S rRNA genes (16SV34). Fifteen µL of Phusion® High-Fidelity Polymerase Chain Reaction (PCR) Master Mix (New England Biolabs, Ipswich, MA, USA), 0.2 µM of primers, and 10 ng of template DNA were used for the polymerase chain reaction (PCR) analysis. The thermal cycling protocol involved a one-minute initial denaturation at 98°C, thirty cycles of denaturation at 98°C for ten seconds, annealing at 50°C for thirty seconds, and elongation at 72°C for thirty seconds and 72°C for five minutes.

Specified regions were amplified by PCR utilizing certain primers that link to barcodes. The PCR products were combined with an equal volume of 1X loading buffer that contains SYBR green. To detect the results, the electrophoresis on a 2% agarose gel was run. Then, the PCR product mixture was purified with a Universal DNA Purification Kit (Tian Gen). The precise number of PCR products from each sample was combined, end-repaired, A-tailed, and then further ligated using Illumina adapters. The NEB Next® Ultra™ II FS DNA PCR-free Library Prep Kit (New England Biolabs) was utilized to create sequencing libraries. The libraries were examined using a bioanalyzer to detect size distribution and Qubit and real-time PCR for quantification. In order to produce 250 bp paired-end raw reads, libraries were sequenced on an Illumina paired-end platform.

Gut Microbiota Analysis

The sequence analysis was done with Uparse software (Uparse v7.0. 1001, *http://drive5.com/uparse/*). Sequences

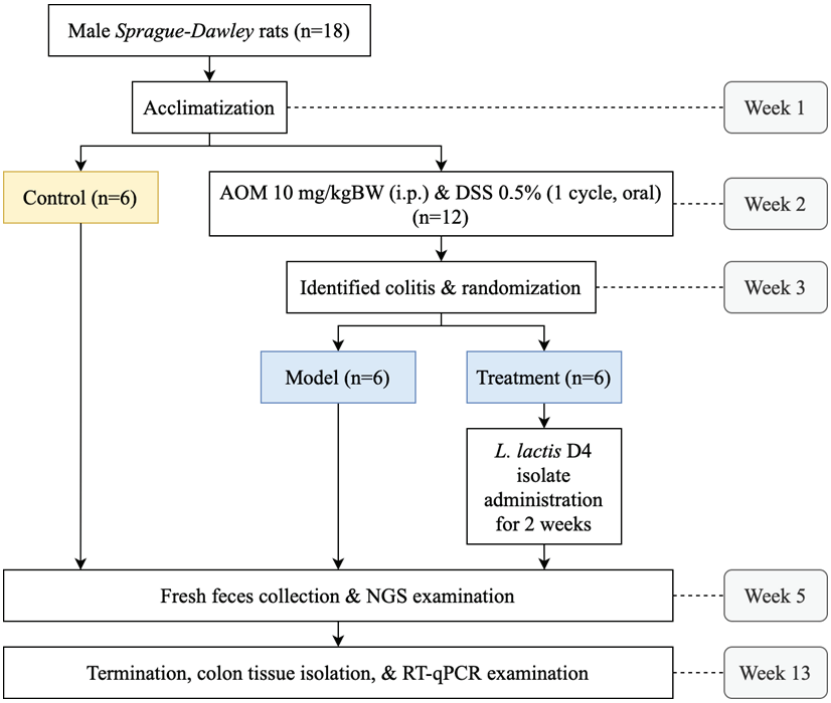

Figure 1. Experimental design chart. Control is the untreated group, Model is the group induced with AOM+DSS only, and Treatment is the group induced with AOM+DSS and given *Lactococcus lactis* D4 isolate.

that shared 97 percent or more similarities were grouped together into operational taxonomic units (OTUs). For additional annotation, representative sequences for every OTU were screened. Each representative sequence's taxonomic data was annotated using the Mothur Algorithm and the Silva Database (*http://www.arb-silva.de/*). Multiple sequence alignments were carried out using the MUSCLE software (Version 3.8.31, *http://www.drive5.com/muscle/*) in order to investigate the evolutionary relationships between various OTUs and the variations in the dominant species in various samples (groups). OTUs' abundance information was normalized using a standard sequence number corresponding to the sample with the most miniature sequences. Data from OTU cluster analyzed for alpha and beta diversity analysis.

Alpha diversity was a measurement of species diversity within a community or sample. It indicates the internal diversity of a community by showing how many species are present and how evenly they are distributed. This measure analyzes the complexity of species diversity for a sample through 6 indices. We used the Simpson and Shannon indexes to identify community richness and diversity. The Shannon index is a tool to evaluate the diversity of species in a community. When the Shannon index value is higher, it indicates greater diversity, which means that all species are distributed more evenly. According to a recent study, the treatment group had the highest diversity, while the positive control group had the lowest diversity. Another index that can be used for this purpose is the Simpson index, which is the opposite of the Shannon index. A lower value of the Simpson index indicates greater diversity, and its value ranges from 0 to 1. A value closer to 1 means that a particular species dominates the community, while a value closer to 0 means that all species in the community are more diverse. All these indices were calculated with QIIME (Version 1.9.1) and displayed with R software (Version 4.0.3).

The differences in sample species complexity were assessed using beta diversity analysis. Beta diversity refers to the extent of similarity in community patterns among three groups. When comparing groups, a value close to zero indicates a stronger similarity in the microbiota patterns of the two groups. Using QIIME software (Version 1.9.1), beta diversity on both weighted and unweighted UniFrac was computed.

Reverse Transcription Quantitative PCR (RT-qPCR) Analysis of Proinflammatory Cytokines

The expression of proinflammatory cytokines analyzed using the RT-qPCR method were transforming growth factor (TGF)-β, nuclear factor kappa-B (NF-κB), IL-6, and tumor necrosis factor (TNF)-α. RNA was isolated from colon tissue using GENEzol™ reagent (Geneaid, New Taipei City, Taiwan). RNA was synthesized into cDNA at 1000 ng/μL using the SensiFAST™ cDNA Synthesis kit (Bioline, London, United Kingdom). Gene amplification was performed by RT-qPCR method using SensiFAST™ SYBR® No-ROX kit (Bioline) with specific primers of TGF-β, NF-κB, IL-6 and TNF-α (Table 1). The gene expression data obtained were normalized using the Livak method ($2^{-\Delta\Delta CT}$).

Statistical Analysis

The data obtained were tested using Kruskal-Wallis and *post hoc* test using Mann-Whitney. The limit of significance was $p<0.05$ with 95% confidence interval. The correlation between the relative abundance of phylum-level bacteria and the ratio of proinflammatory cytokine expression was analyzed using the Spearman rank correlation test. Data analysis was performed using SPSS version 25 (IBM Corporation, Armonk, NY, USA) and visualized with GraphPad Prism version 10 (GraphPad Software, Boston, MA, USA).

Results

L. lactis D4 Modulated Gut Microbiota Composition in AOM and DSS-induced Colitis Rats

Figure 2 depicted the varied composition of microbiota among three groups in the tested feces. At the phylum

Table 1. Primers used for RT-qPCR assay.

Cytokines	Forward (5'-3')	Reverse (3'-5')
TGF-β	CAAGGAGACGGAATACAGGGC	CTCTGTGGAGCTGAAGCAGTAG
NF-κB	AAGCAGGAAGATGTGGTGGAGG	GAGTAGGACCCCGAGGATTTTATC
IL-6	CAGCGATGATGCACTGTCAG	CTCCAGGTAGAAACGGAACTCC
TNF-α	CCAGACCCTCACACTCAGATC	CAGCCTTGTCCCTTGAAGAGA
GADPH	CGGTGTGAACGGATTTGGC	CTCGCTCCTGGAAGATGGTG

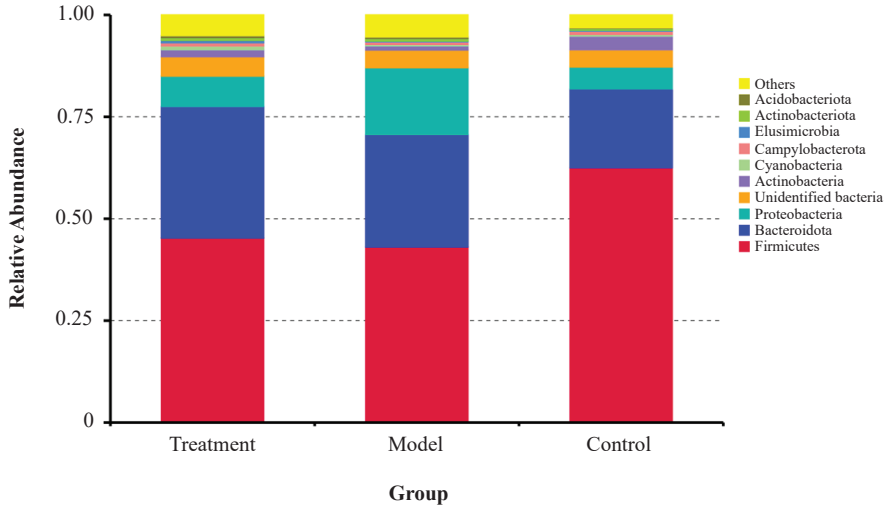

Figure 2. Effect of *L. lactis* D4 to the gut microbiota composition based on the phylum level.

level, Firmicutes, Bacteroidetes, and Proteobacteria were the dominant phyla in each group. In the control group, Firmicutes was the predominant phylum with more than 50% account. The administration of AOM and DSS caused significant changes in the composition of the gut microbiota by killing beneficial bacteria, as evidenced by the decrease in the number of Firmicutes and an increase in the number of pathogenic phyla such as Proteobacteria and Bacteroidetes. The treatment group, which was given the *L. lactis* D4 isolate, was able to restore the balance of microbiota by increasing the phyla of Firmicutes and Bacteroidetes while decreasing Proteobacteria compared to the AOM and DSS group. Genus level in the top phylum was drawn in heatmap to check whether the samples with similar processing are clustered or not (Figure 3). Similarity of the gut microbiota also depicted from beta diversity heatmap (Figure 4). UniFrac ponderated value between the control and treatment groups was 0.400, while the value between the control and colitis-induced (model) groups was 0.480. This meant the composition of the gut microbiota was considerably more akin to the control group following treatment with *L. lactis* D4 as compared to the model groups induced with AOM and DSS. Meanwhile, the statistical index of alpha diversity at 97% clustering threshold was shown in Table 2.

Based on the analysis at the genus level, bacteria from the Firmicutes phylum were found to be dominant in each group. However, their numbers decreased after the administration of AOM and DSS. Although all the microbes belonged to the same phylum, the dominating microbes came from different genus. In the control group, the Firmicutes phylum was dominated by *Colidextribacter*, *UCG-005*, *Lactobacillus*, *Limosilactobacillus*, and *Lachnospiraceae NK4A136* group. This phylum was still dominant in the colitis-induced and treatment groups as well, but the genus of microbes changed. In the colitis-induced group, the dominant genera were *Faecalibacterium*, *Lachnospiraceae ND3007* group, *Erysipelotrichaceae*, and *Catenibacterium*. Whereas, in the treatment group, the genera *Anaerovibrio*, *Lactococcus*, *Ruminococcus*, and *Anaerostipes* were dominant. A similar trend was observed in other phyla such as Bacteroidetes and Proteobacteria.

L. lactis D4 Affected Inflammatory Cytokines in AOM and DSS-induced Colitis Rats

The results showed that the expression of TGF-β, NF-κB, and TNF-α tended to increase after the administration of *L. lactis* D4 while the expression of IL-6 decreased but the level was not statistically significant (Figure 5). AOM and DSS had different effects on these four cytokines, NF-κB and TNF-α gene expression tended to be similar or increase compared to the control group, while TGF-β and IL-6 gene expression was decreased. Interestingly, *L. lactis* D4 therapy further suppressed IL-6 cytokine production, while TGF-β expression increased, similar to the other two cytokines. This suggested that *L. lactis* D4 may have a specific effect on IL-6 compared to other proinflammatory cytokines such as TGF-β, NF-κB, and TNF-α.

Correlation Analysis of *L. lactis* D4 Treatment Effect on Gut Microbiota and Cytokines

To investigate the correlation between the composition of gut microbiota and levels of proinflammatory cytokines, the researchers employed Spearman's rank correlation test to determine how the process of intestinal adaptation occurs after exposure to certain carcinogens or materials, and how *L. lactis* D4 can help to mitigate further intestinal damage, treat dysbiosis, and restore microbiome balance.

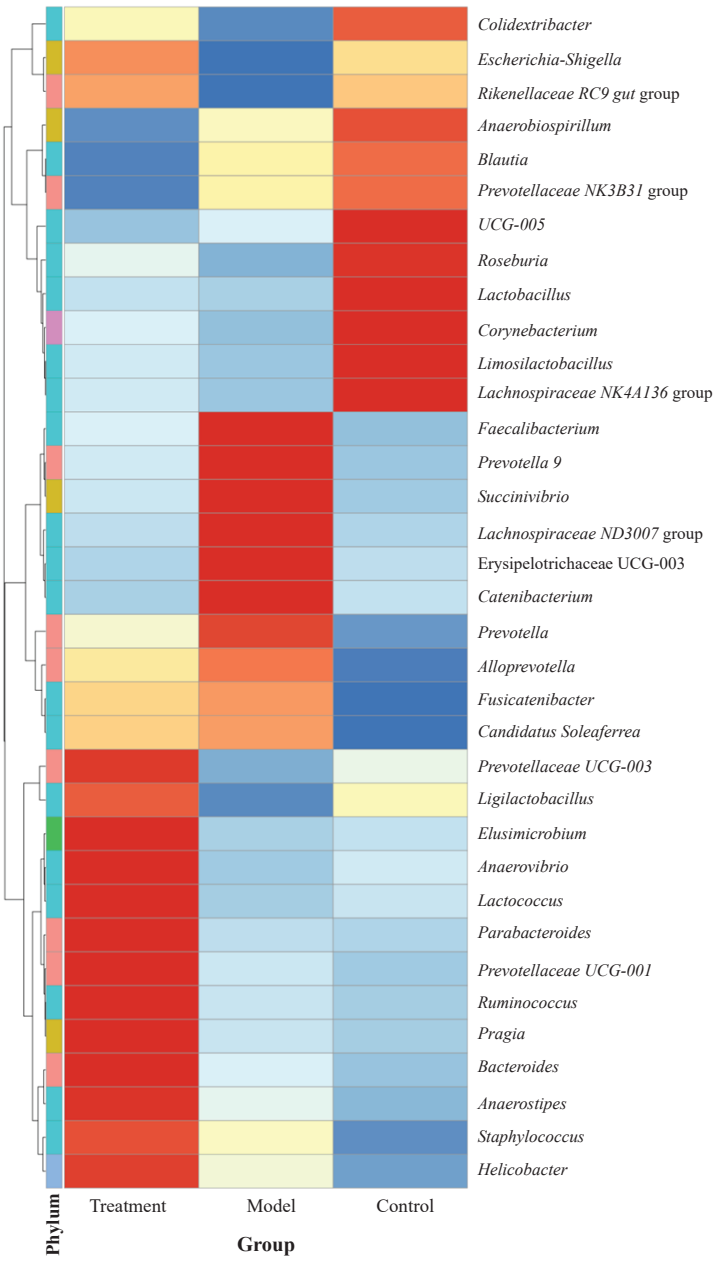

Figure 3. Taxonomic abundance cluster heatmap based on genus level. The number (1 to -1) indicates amount of the microbiota in the phylum. Higher number indicates growth of microbiota and was shown by more reddish color while lower number indicates suppression of microbiota and was shown by more bluish color.

Figure 6 illustrated that after given *L. lactis* D4 in the treatment group, Firmicutes and Actinobacteria phylum dominated composition of the gut microbiota (marked by red color). The increased expression of proinflammatory cytokines such as TGF-β and TNF-α was positively correlated (correlation coefficient; 0.886 and 0.943 consecutively) with microbiota from the phylum Actinobacteria, while the increase in NF-κB was negatively correlated (correlation coefficient; -0.829) with the phylum Elusimicrobia. Furthermore, the study demonstrated that the decrease in IL-6 expression in the treatment group was positively correlated (correlation coefficient; 0.943) with microbiota derived from the phylum Actinobacteria (p<0.01).

Discussion

The study utilizes the benefits from a new strain of *L. lactis* derived from *dadih*, which was *L. lactis* strain D4. The results of this study showed that Firmicutes phylum dominated in the control group. The number of Firmicutes phylum was found decreased after AOM and DSS induction and restored after *L. lactis* D4 application. The gut has more than 1000 species with six dominant phyla, including Firmicutes. The data shows that Firmicutes have a quantitative range of 20.5% to 80% in the gut of healthy adult humans.(15) The Firmicutes phylum is the normal gut flora of gram-positive bacteria and plays a role in the

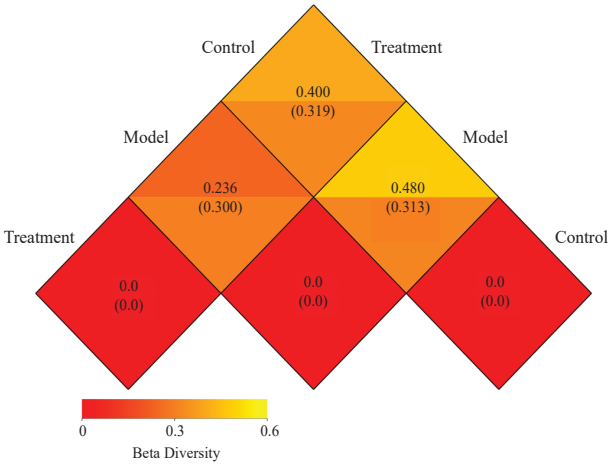

Figure 4. Beta diversity heatmap between three groups of experiment. A value close to zero (0 to 0.6) and reddish color indicates a stronger similarity in the microbiota patterns of the two groups in the heatmap.

fermentation of carbohydrates into short-chain fatty acids (SCFA).(16)

This study highlighted an increase in Bacteroidetes in the treatment group compared to the model group, emphasizing the role of this phylum alongside Firmicutes in maintaining gut flora balance. Conversely, an elevation in Proteobacteria, often associated with gut microbiota instability and inflammation in IBD, was noted in the model group. This is consistent with their characterization as markers of dysbiosis and their increased prevalence in inflammatory conditions.(17,18)

L. lactis D4 given to colon and rectum that have inflammation as part of the mechanism of colorectal carcinogenesis through induction of AOM and DSS, can restore the balance of intestinal microbiota by maintaining beneficial microbiota, specifically microbes from the Firmicutes phylum and increasing the number of microbes from the Bacteroidetes phylum and suppressing pathogenic microbiota, especially those from the Proteobacteria phylum. However, the microbiota is different from the control group at the genus level. This means that *L. lactis* D4, either directly or indirectly through its metabolite products, causes overgrowth of several microbial genera and inhibits the growth of other microbes, both beneficial and pathogenic microbes as already shown in the heatmap in Figure 3.

Table 2. Statistical index of alpha diversity at 97% clustering threshold.

Sample	Observed Species	Shannon	Simpson
Treatment	1839	7.519	0.981
Model	1823	6.837	0.968
Control	1652	6.949	0.963

Probiotics play a significant role in the regulation of microbiota homeostasis. The findings of this study showed that there were 155 identical species between the treatment and control groups. Beta diversity heatmap analysis showed that the pattern of gut microbiota in the control group was more similar to that of the treatment group than that of the model group. Microbiota regulation through probiotic administration involves several mechanisms. First, probiotics improve normal gut flora's distribution by stimulating these bacteria's growth. Interventional studies related to *Lacticaseibacillus casei* administration showed that probiotics not only increase the number of *Lactobacillus* sp. but also other normal flora, such as *Roseburia*, *Coprococcus*, and *Eubacterium rectale* while inhibiting the growth of pathogenic microbiota such as *Blautia* and *Ralstonia*. Secondly, probiotics regulate gut homeostasis through competitive exclusion, *i.e.*, competition in ecology and nutrients to promote the colonization of desirable gut bacteria and prevent the growth of pathogenic bacteria. Probiotics can maintain microbiota homeostasis by regulating colonic conditions through the secretion of metabolites.(19)

Strains within the *L. lactis* group are known to produce bacteriocins. These bacteriocins, typically small, positively charged cationic peptides, exert their antimicrobial activity against target cells by forming pores in their membranes, leading to cell death. The spectrum of activity extends to both closely related strains and pathogenic bacteria, including *Salmonella*, *Staphylococcus*, *Listeria*, *Clostridium*, and *Enterococcus*. Notably, some bacteriocins demonstrate efficacy against viral infections caused by Rotavirus, Norovirus, and Adenovirus. The gut microbiota plays a critical role in maintaining human health through its influence on various bodily functions. Probiotic and their associated bacteriocins hold promise for gut microbiota modulation through their combined antimicrobial and immunomodulatory properties. This potential application could contribute to the restoration of a balanced gut microbial community and enhance host immunity.(20)

The study suggests that *L. lactis* D4 has a positive impact on the regulation and maintenance of intestinal microbiota by reducing inflammatory cytokines such as IL-6 but instead these probiotic increased the level of other pro-inflammatory cytokines (TGF-β, TNF-α, and NF-κB). Addition *L. lactis* D4 to the treatment group, induced

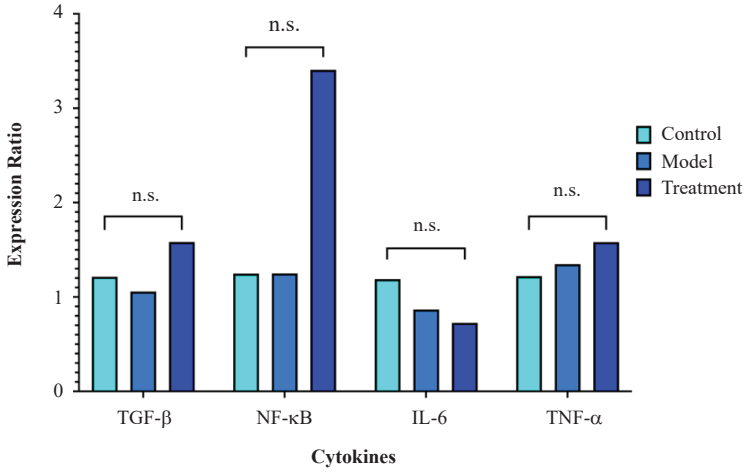

Figure 5. Comparison of proinflammatory cytokines ratio between groups. *p<0.05 considered as significant; n.s.: not statistically significant.

immune system to ameliorate inflammation and injury of the colon that caused by AOM and DSS. This point showed that *L. lactis* D4 has immunomodulatory effect.

The results of this study also showed that there is a positive correlation between the levels of inflammatory cytokines and phylum Actinobacteria. Although Actinobacteria is a minority group of commensal bacteria, it plays a crucial role in developing and maintaining the intestinal homeostasis. Its involvement is thought to be in regulating intestinal permeability, the immune system, metabolism, and the brain-gut axis.

Moreover, *L. lactis* D4 has a synergistic effect with phylum Actinobacteria. The study found that it reduced the levels of Proteobacteria and unidentified bacteria in rats with colitis, implying that it can inhibit other bacterial growth, especially pathogenic microbes. This effect is possibly due to the presence of nisin, a metabolite product of *L. lactis*, known for its antimicrobial effects against pathogenic bacteria and viruses. As a result, there is a decrease in the number of pathogenic bacteria that cause colitis. These findings are supported by existing literature. (21-23) However, further research is needed to evaluate the mechanism of nisin to inhibit the pathogenic bacterial overgrowth and reduce inflammation.

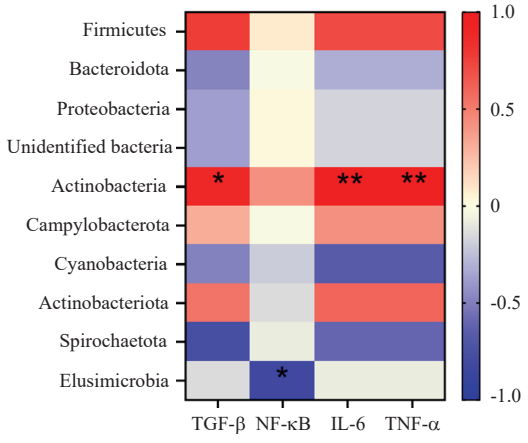

Figure 6. Spearman's rank correlation between gut microbiota and cytokines in the treatment group. The colors and numbers indicate amount of microbiota and level of cytokines. The red color shows higher number than the blue color. *p<0.05; **p<0.01.

Conclusion

L. lactis D4 has a beneficial effect in decreasing pro-inflammatory cytokines and is able to restore gut microbiota composition. Thus, *L. lactis* D4 might be used as a therapeutic agent for IBD. However, more studies are needed to evaluate the benefit and adverse effects of *L. lactis* D4.

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Authors Contribution

RS, AV, ED, JS were involved in concepting and planning the research. RS performed the data acquisition/collection, calculated the experimental data, performed the analysis, drafted the manuscript and designed the figures, AV, ED, JS aided in drafted the manuscript and interpreting the results. All authors took parts in giving critical revision of the manuscript.

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