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Original Article

Effects of the multi-strain probiotic preparation LabMix on some immune indices and intestinal microbiota in an antibiotic associated diarrhea rat model

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Abstract

Diarrhea is a side effect of antibiotic misuse and is frequently associated with intestinal inflammation and imbalanced gut microbiota. Many studies have demonstrated that probiotics can exhibit potential to mitigate the effects of antibiotic-associated diarrhea (AAD). In this study, we employed Lincomycin to induce AAD in the rats and subsequently assessed the impact of the multi-strain probiotic preparation LabMix on this model. The rat groups, including healthy control rats, AAD-induced rats, AAD rats with no treatment (natural recovery rats), and AAD rats treated by LabMix preparation, were evaluated regarding the general assessments, some immune indices, and intestinal microbiota analysis. The results revealed that the LabMix preparation considerably lowered the effects of the antibiotic regarding the diarrhea score and the thickness of the ceca in the rats treated by LabMix preparation. Additionally, the LabMix preparation reduced inflammatory cytokines, including TNF-α, and IL-6, while increasing the IgA in sera and in intestinal mucosae. Furthermore, it altered the compositions and abundance of intestinal bacteria of the rats. In particular, the AAD rats treated by LabMix preparation decreased the levels of potentially harmful genera such as *Bacteroides*, *Escherichia-Shigella*, and *Pseudomonas*. They also increased the levels of beneficial genera including *Lactobacillus*, *Bacillus*, *Romboutsia*, and *Clostridium innocuum*. In general, the multi-strain probiotic preparation LabMix showed the effective mitigation and the improvement of the intestinal microbiota of the AAD rat model.

Keywords: Antibiotic Associated Diarrhea (AAD), Cytokine, LabMix, Microbiota, Probiotic

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Introduction

Antibiotics are frequently utilized in the treatment of various illnesses caused by bacteria (Larcombe et al., 2016). However, incorrect antibiotic usage causes substantial problems including diarrhea, a higher incidence of antibiotic-related illnesses, and antibiotic resistance, which have prompted considerable clinical issues (Larcombe et al., 2016; Mekonnen et al., 2020). Antibiotic-associated diarrhea (AAD) is a common adverse side effect of noncompliance and abuse of antibiotics. The improper use of antibiotics in treatment disrupts the gut microbiota, decreases the abundance, diversity, and uniformity of the gastrointestinal flora, and reduces the percentage of friendly bacteria, while increasing the number of disease-causing pathogens like *Candida*, *C. difficile*, and other opportunistic pathogens like *K. oxytoca*, *K. pneumonia*, *C. perfringens*, *S. aureus*, and *K. oxytoca* (Willing et al., 2011; Bartlett and Gerding, 2008). Therefore, decrease in antibiotic side effects become one interesting issue for research (Gresse et al., 2017). Probiotics are defined as living bacteria that, when given in the right amounts, benefit the host organisms (WHO/FAO, 2001). Probiotics have been found to restore disturbed gut microbiota and suppress infections, and they have been employed in many clinical trials to prevent AAD (Guo et al., 2019). Lactic acid bacteria (LAB) are widely used as probiotic bacteria. *Lactobacillus* is one of the LAB genera that consists of most GRAS species, and their numerous strains are used in food microbiology and human nutrition (Pessione, 2012). The multi-strain probiotic preparation LabMix is a powder of three bacteria strains, including *Lactobacillus acidophilus* LA 304.17, *Lactobacillus casei* LC 304.08, and *Bifidobacterium bifidum* BF 304.98. All of the three strains were isolated from Vietnamese healthy people and Vietnamese fermented foods, and they met the *in vitro* requirements according to the FAO/WHO's recommendation for bacterial probiotic strains. The preparation has been tested in rats for acute and semi-permanent toxicity.

The gut bacteria have a vital role in maintaining intestinal homeostasis and human health. In general, gut microbiota contributes around 70% of the immune system. Therefore, alterations in their compositions can cause some disorders like diarrhea and illnesses such as cardiovascular disease, inflammatory bowel disease (IBD), diabetes, irritable bowel syndrome (IBS), and allergies (Ott et al., 2004). Many studies have shown the effects of probiotics on gut microbiota (Kim et al., 2019). Before being used for humans, pharmaceuticals in general and probiotic products in particular should be tested in animal models, and mice and rats are frequently used to examine the effects of these products (WHO/FAO, 2001). This study was conducted to assess the effects of LabMix preparation for general assessments, some immune indices, and the observable changes of intestinal microbiota in the AAD rat model.

Material and Methods

Material

The multi-strain probiotic preparation LabMix, containing *Lactobacillus acidophilus* LA 304.17, *Lactobacillus casei* LC 304.08, and *Bifidobacterium bifidum* BF 304.98 with a density of 3×10^9 CFU/g for each strain, was manufactured at the GMP-certified factory of Nam Viet Biotechnology Joint Stock Company. The product met the basic standards provided by the Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi.

Lincomycin, with the registration code of VD-29184-18 and expiration date of September 25, 2024, manufactured on September 25, 2021, (Domesco, Vietnam) was employed for inducing diarrhea in the rats.

Wistar rats weighing 180 ± 20 g, were provided by The Military Medical Academy (Hanoi, Vietnam) under the animal license number 03:2021/VNU-IMBT. Each rat was individually housed within a free-of-pathogens animal room at a controlled temperature of 23 ± 2 °C and relative humidity of 60 ± 5%, with unrestricted access to both food and drink.

Ethics statement

The conducted procedures and utilization of animals adhered to the regulations stipulated by both the Vietnamese Laboratory Animal Law and the Guidelines for the Caring and Use of Laboratory Animals.

Experimental design

After adapting to the environment of the animal laboratory, a total of 32 rats were randomly assigned into four groups with 8 animals per group. Based on the results of the study of Guo (2021) and characteristic of the strains in the LabMix preparation, these groups were as follows: i) Control group (CG): rats were administered 0.5 ml /100 g /24h distilled

water; ii) AAD group (AD): rats were induced to have diarrhea by oral administration of Lincomycin at the dosage of 5 g/ kg /24h for 4 days and on the 5th day, the rats were killed to obtain blood and ceca samples for subsequent analysis; iii) natural recovery group (NR): rats were drank 0.5 ml /100 g/ 24h distilled water for 5 days after being induced to have diarrhea, similar to the AD group; iv) LabMix treatment group (LA): rats were orally administered LabMix preparation at the dosage of 2.52×10^9 CFU/kg/24h for 5 days after being induced to have diarrhea as the AD group (Guo, 2021) . On the 9th day, the rats from the CG, NR, and LA groups were anesthetized using ether. Their cardiac sera were collected, centrifuged, and stored at -80°C. Additionally, the cecal specimens were also gathered and preserved at -80°C.

General assessments

The body weights of the rats and diarrhea scores were assessed. Throughout the experimental period, the rats in all groups were weighed every day. Diarrhea scoring was conducted based on the following criteria: i) 0 points: for healthy rats; ii) 1 point: for rats displaying a normal mental state, accompanied by loose and non-adherent perianal stools; iii) 2 points: for rats displaying a bad mental state, adhesion stool around the anus, inappetence, and weight loss (Ren et al., 2022).

Histopathological analysis

The organs of the rats, including the liver, kidney, spleen, and cecum were weighed, and the cecal specimens were fixed in 10% formalin for microscopic evaluation. A standard ruler according to each objective 4X, 10X, and 20X was used to measure the thickness of the cecum mucosa. The mucosal thickness was calculated from the superficial epithelial cells to the mucosal muscle of the cecum.

Cytokine and immunoglobulin analysis

After adding 1 ml of tissue extraction reagent I, 0.1 g of intestinal mucosal tissue in an Eppendorf tube was homogenized by a Wiggens D-5000 machine (Wiggens, Germany), and subsequently centrifuged at 10000 rpm for 5 minutes to collect the supernatant for analyzing IgA of intestinal mucosal tissue. IgA of sera, IgA of intestinal mucosal tissue, and blood cytokine index (IL6, TNF- α) were quantified utilizing the ELISA kit provided by Thermo Fisher Scientific.

DNA extraction and sequencing

Total bacterial genomic DNA was extracted from cecal stool samples using the QIAamp fast DNA stool mini kit (code 51604, Quiagen, Germany), following the manufacturer's protocols. The DNA extracts were checked for their integrity using a 1% agarose gel, and the concentrations were determined using a Nanodrop spectrophotometer (Thermo Fisher, USA). After quality and quantity checking, DNA samples were sent for sequence targeting the V3-V4 regions of the 16S rDNA gene by using the Illumina MiSeq sequencing platform.

Intestinal microbiota analysis

Following the application of the DADA2 software to eliminate chimeras and sequences of unknown length, and qualitative assessment of raw fastq sequence data using Q scores, the collected sequences were subjected to analysis using Qiime2 (version 2023.5). From a pool of 32 DNA bacterial samples extracted from the rats, a total of 5,926,022 high-quality sequences were obtained after fliltering, exhibiting a Q score of 30 and an average length of 372.5 bp. The mean read count per sample was 185,188 ± 49,751. Amplicon Sequence Variants (ASVs) with 99% similarity were utilized to generate a feature table, subsequently used to construct the microbial composition profile of each sample by referencing the Silva database. Prism softwere (version 9) was utilized to assess alpha-diversity indices such as Chao 1, Simpson, Shannon, and Evenness. Beta-diversity was analyzed using weighted UniFrac distances with the results being visualized through Principal Coordinate Analyses (PCoA) (Huse SM et al., 2008). Qiime2 was used to do a comprehensive statistical analysis of the bacterial community at both the genus and phylum levels.

Statistical analysis

All data analyses were conducted using SPSS 26.0 (IBM), and the outcomes were presented as mean values accompanied by their corresponding standard deviation (M ± SD). For multi-group analyses, the ANOVA test was used to ascertain statistical significance, while the Wilcoxon test was utilized for pairwise comparisons. Statistical significance was deemed for p-values <0.05 or p-values <0.001.

Results

General and histopathology assessments

To comprehensively evaluate the health and physiological changes, a range of parameters,

including diarrhea score, body weight, caecum thickness, and the visceral weight of organs such as the liver, kidney, and spleen, were examined across all rat groups. The progression of diarrhea in the rats exhibited significant growth ($p<0.05$), reaching its peak score on the fourth day and persisting until the fifth day for the LA group, and even extending to the sixth day for the NR group **(**Figure 1A**)**. The presence of loose feces and a red inflamed anus indicated a successful model of AAD in rats by using Lincomycin (Yang X et al., 2021). During the first four days of the experiment, rats in the three groups (AD, NR, and LA) dramatically lost their body weights, but in the following five days the rat weights in both the NR and LA groups increased modestly **(**Figure 1B**).** The weights of the liver, kidney, and spleen of the rats did not differ for the 4 groups ($p>0.05$) **(**Figure 2A**).** However, the ceca in the three groups (CG, NR, and LA) were thicker than those in the AD group ($p<0.05$). In addition, the thickness of the ceca in the CG and LA groups did not show significant differences **(**Figure 2B**).**

The cecum image of the CG rat group was normal with tiny and regular nuclei of epithelial cells **(**Figure 3A**).** In the LA group, the tiny and regular nuclei of epithelial cells were also observed, but the capillary stroma were somewhat clogged **(**Figure 3D**).** However, there were foci of inflammatory cells creating big and tiny lymphoid follicles in the mucosa and submucosa in the AD and NR groups **(**Figure 3B, 3C**)**. In addition, mild edema and inflammatory infiltrates were also observed in both groups. Moreover, in the AD group, inflammatory lesions promoted surface gland degeneration, and many of them were atrophied.

Inflammatory cytokines and Immunoglobulin changes

The AD and NR groups showed higher IL-6 and TNF-α levels than those in the CG and LA groups, and these differences were statistically significant with $p<0.05$ (Figure 4A, 4B). In contrast to cytokines, IgA concentrations in sera and intestinal mucosae of the AD and NR groups declined considerably compared to those of the LA and CG ($p<0.05$) (Figure 5A, 5B).

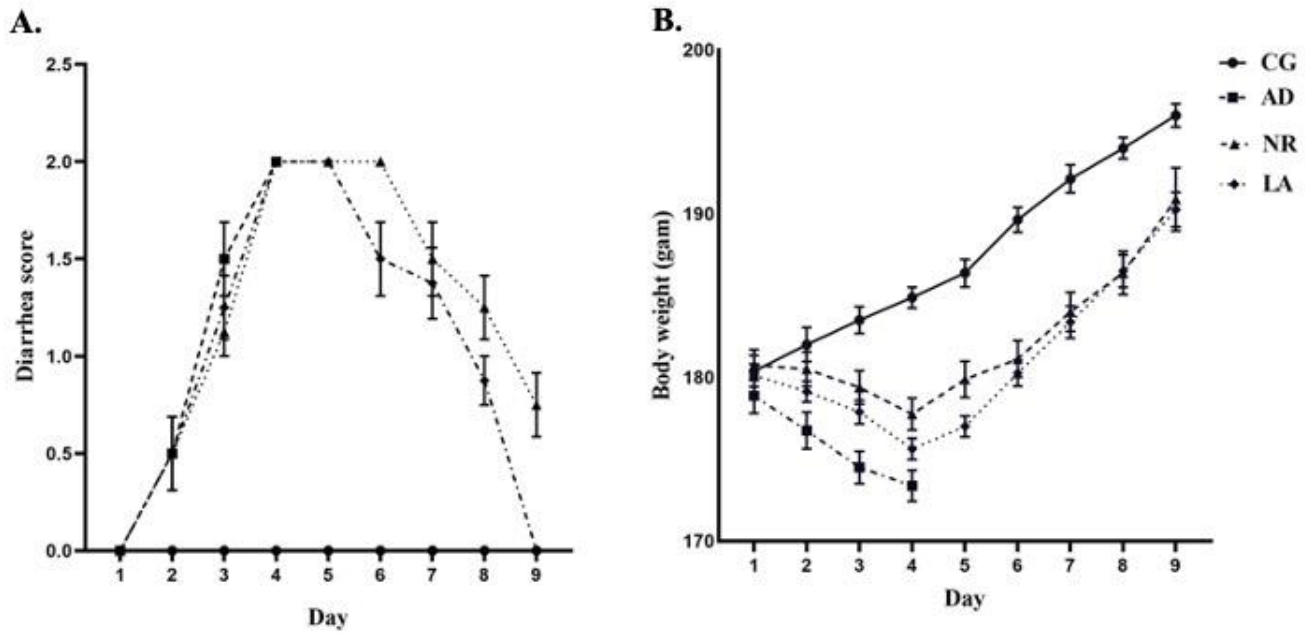

Figure-1. Diarrhea score (A) and body weight (B) of the rats.

CG, control group; AD, AAD group; NR, natural recovery group; LA, LabMix treatment group. The Mean ± SD is used to represent the values.

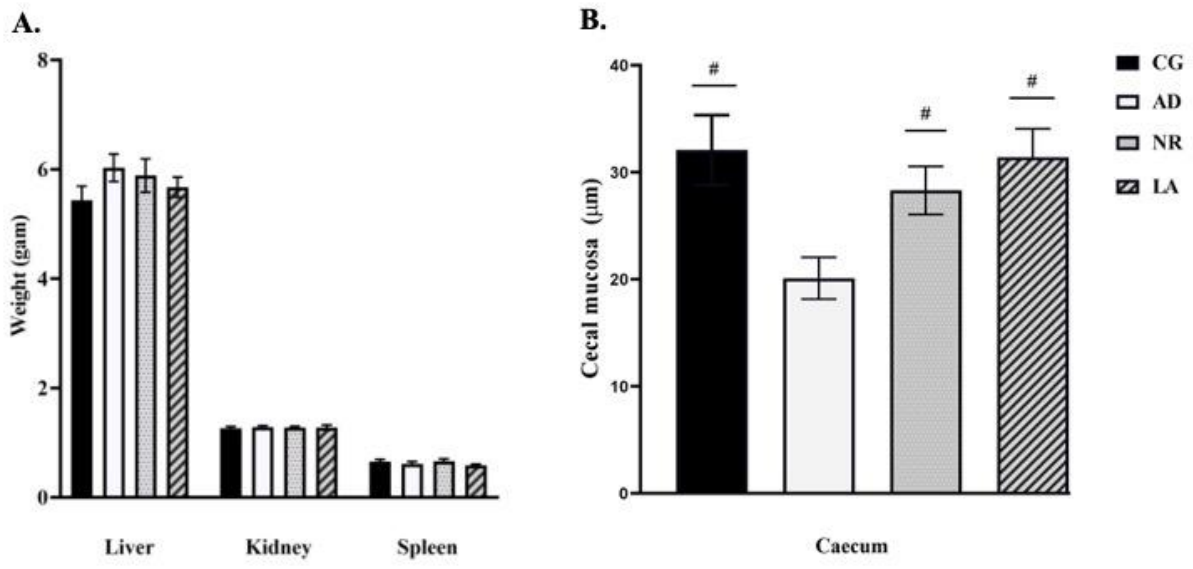

Figure-2. The weight of liver, kidney, spleen (A), and cecal mucosa thickness (B) of rats

CG, control group; AD, AAD group; NR, natural recovery group; LA, LabMix treatment group. The M ± SD is used to represent the values, #p< 0.05 compared with DA.

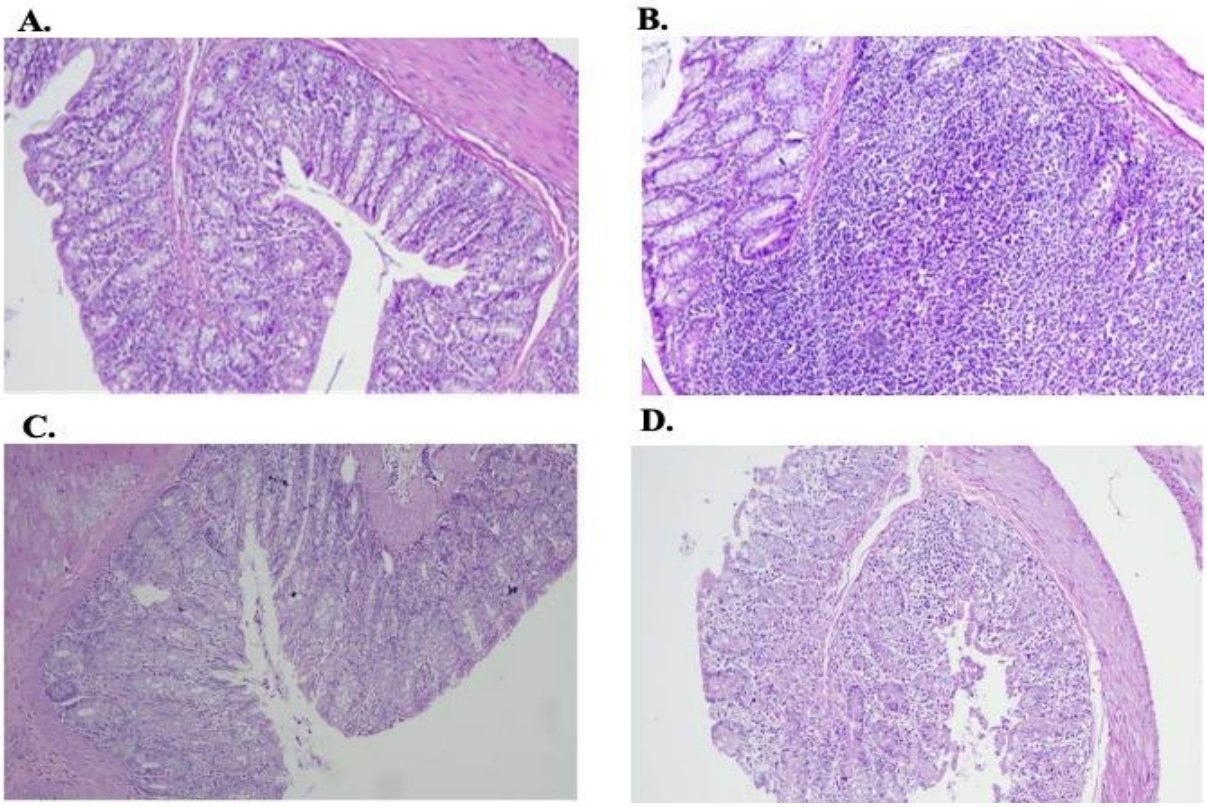

Figure-3. Microscopic image of the rat ceca

Control group (A); AAD group (B); natural recovery group (C); LabMix treatment group (D).

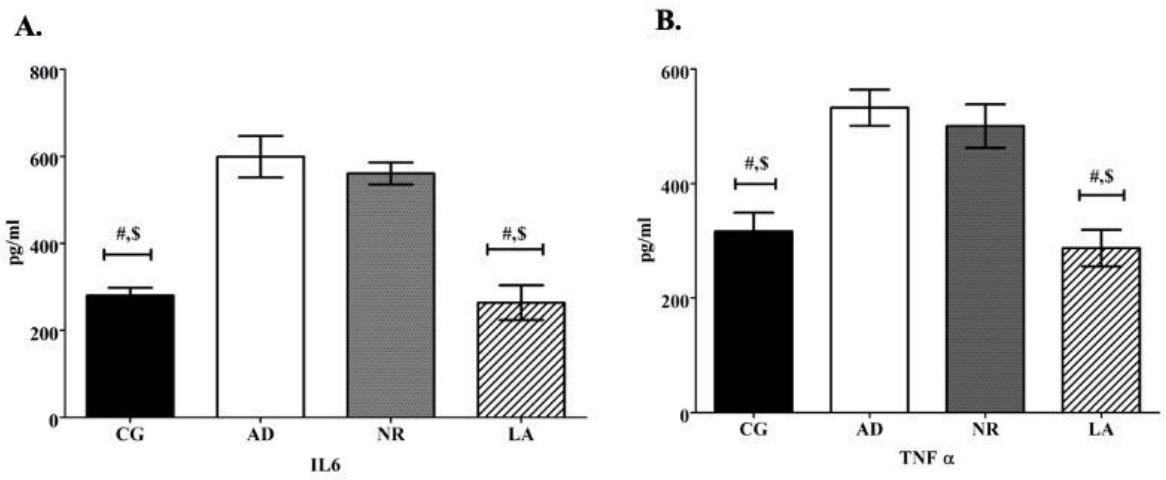

Figure-4. Levels of pre-inflammatory cytokines IL-6 (A); TNF-α (B) of rats

CG, control group; AD, AAD group; NR, natural recovery group; LA, LabMix treatment group. The Mean ± SD is used to represent the values, (#) p<0.05 compared with AD, ($) p<0.05 compared with NR.

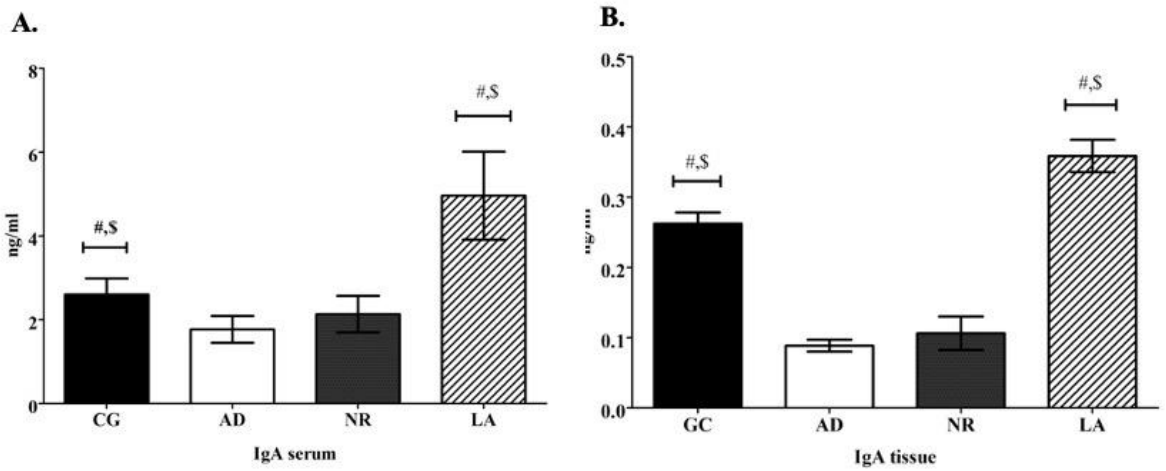

Figure-5. Levels of IgA in serum (A), and IgA in intestinal mucosae (B) of rats

CG, control group; AD, AAD group; NR, natural recovery group; LA, LabMix treatment group. The Mean ± SD is used to represent the values, (#) p<0.05 compared with AD, ($) p<0.05 compared with NR.

Diversity of intestinal microbiota of the rats

The effects of LabMix preparation on the diversity of intestinal microbiota in the rats were evaluated for alpha diversity, and beta diversity of the rat groups. Regarding alpha diversity, the Simpson, Shannon, Evenness, and Chao 1 indices were examined. The Simpson index with the values of 0.95 ± 0.03; 0.74 ± 0.05; 0.86 ± 0.03, and 0.87 ± 0.08 for the CG, AD, NR, and LA groups, respectively, showed significant differences between the CG and the 3 remaining groups, i.e., the AD, NR, and LA groups, and significant difference between the LA and AD groups **(**Figure 6A**).** The Shannon and Evenness indices showed remarkable differences when comparing the CG and LA groups with the AD and NR groups (p<0.05) (Figure 6B and Figure 6C**).** In addition, there were no significant differences for the LA and CG groups regarding the Evenness index (p>0.05) (Figure 6C**).** The Chao 1 index with the values of 1168.16 ± 555.16; 802.98 ± 933.6; 570.22 ± 296.63, and 1062.10 ± 908.24 for the CG, AD, NR, and LA groups, respectively, showed no significant differences among all the groups (Figure 6D**).**

For beta diversity, the PCoA showed that the CG group was separated from the three remaining groups, i.e., the groups of AD, NR, and LA. Interestingly, the

position of the NR group was between those of the AD and LA groups. The variances of all the samples determined by PC1 and PC2 were 28.86%, and 13.25%, respectively **(**Figure 7**).**

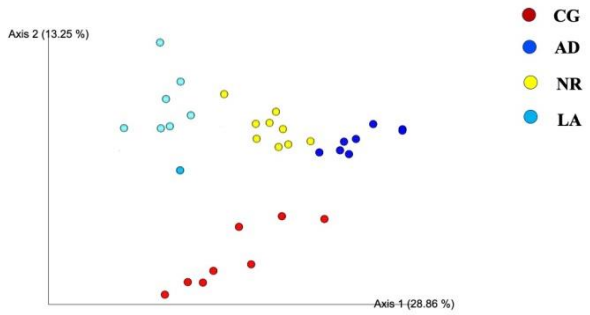

Figure-7. The PCoA graph of the intestinal bacteria in the 4 rat groups
CG, control group; AD, AAD group; NR, natural recovery group; LA, LabMix treatment group

Composition of the intestinal microbiota at phylum and genus levels of the rats

The data were analyzed by comparing the AAD rats treated by LabMix preparation (LA group) with the untreated AAD rats, i.e., the AD and NR groups as well as the rats of the LA group with the healthy rats of the CG group.

The compositions of the gut microbiota at the phylum level of all the rat groups were shown in Figure 8A, in which Bacteroidota, Firmicutes, and Proteobacteria, were the most dominant. In the LA group, the Bacteroidota and Proteobacteria abundance was the lowest (36.90% and 9.39%, respectively), whereas Firmicutes abundance was the highest with the values of 49,61% in comparison with the AD and NR groups. The ratios of Firmicutes and Bacteroidota were 1.34, 0.20, 0.91, and 0.96 in the CG, AD, NR, and LA groups, respectively. Notably, a significant difference was observed between the treated AAD rats (LA group) and untreated AAD rats (AD and NR groups) for the Bacteroidota and Firmicutes abundance. In addition, there was no significant difference between the LA and CG groups for these phyla (Figure 8B, 8C, and 8D).

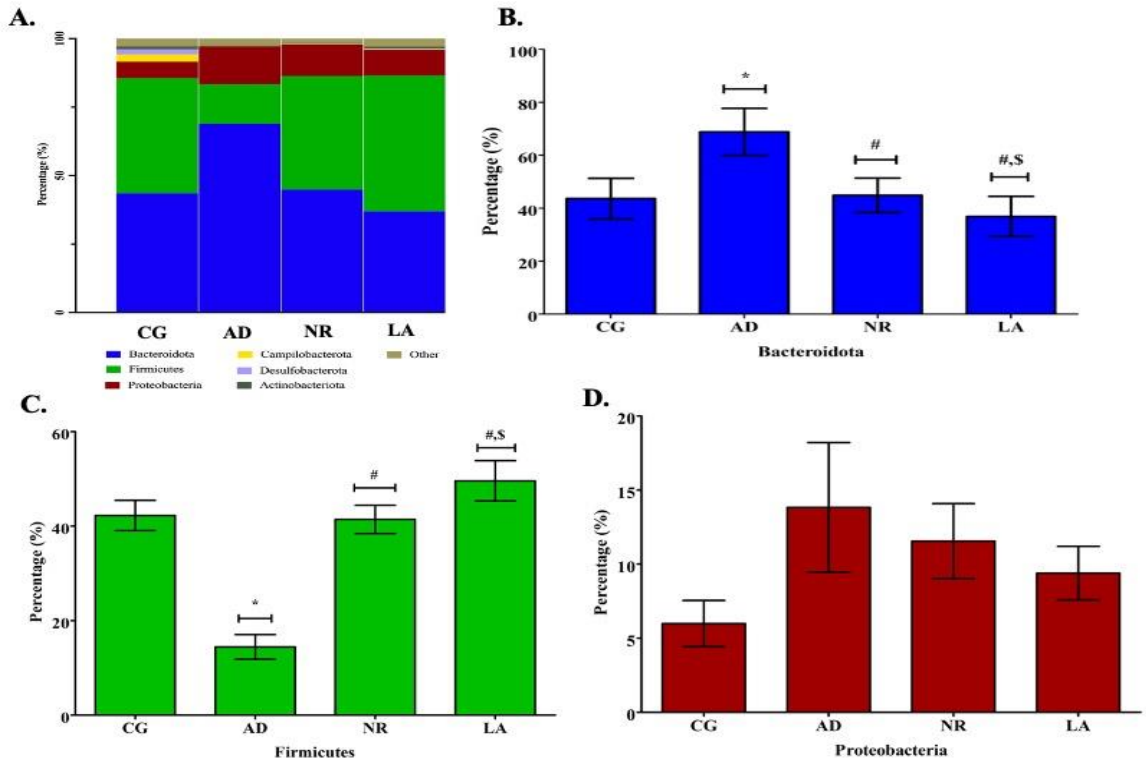

Figure-8. The intestinal microbiota composition at phylum level of 4 rat groups (A), major differences in the levels of Bacteroidota (B), Firmicutes (C), Proteobacteria (D) of the 4 rat groups
CG, control group; AD, AAD group; NR, natural recovery group; LA, LabMix treatment group. The Mean ± SD is used to represent the values. (*) p<0,05 compared with CG, (#) p<0.05 compared with AD, ($) p<0.05 compared with NR

At the genus level, the differential abundance of some other genera, including harmful and beneficial/natural bacteria, was analysed for all the groups (Figure 9A). *Bacteroides*, *Escherichia-Shigella, and Pseudomonas* abundance in the LA group was the lowest with the values of 33.70%, 4.47%, 0.11%, respectively, in comparison with the AD and NR groups. Moreover, *Bacteroides* abundance showed a significant difference between the treated AAD rats (LA group) and untreated AAD rat (AD and NR groups). However, there were significant differences between the LA and NR groups for *Escherichia-Shigella*, and the LA and the CG groups for *Bacteroides* and *Escherichia-Shigella*, and (Figure 9B, and 9C).

In contrast to harmful bacterial genera, some beneficial bacteria showed noticeable increase in the LA group compared to those in the AD and NR groups. Interestingly, the *Lactobacillus*, *Bacillus*, and *Romboutsia* abundance in the LA group was the highest with the values of 8.04%, 0.5% and 1.91%, respectively, in comparison with the AD and NR groups. Moreover, these useful bacterial showed significant differences when comparing the treated AAD rats (LA group) and untreated AAD rats (AD and NR groups), and no significant differences when comparing the LA and the CG groups. (Figure 10A, 10B, 10C). Additionally, *Muribaculaceae*, a natural genus, was dominant only in the CG group and its abundance showed the highest value (22.51%) with a significant difference compared to the remaining groups, i.e., the LA, NR, and AD groups (Figure 10D).

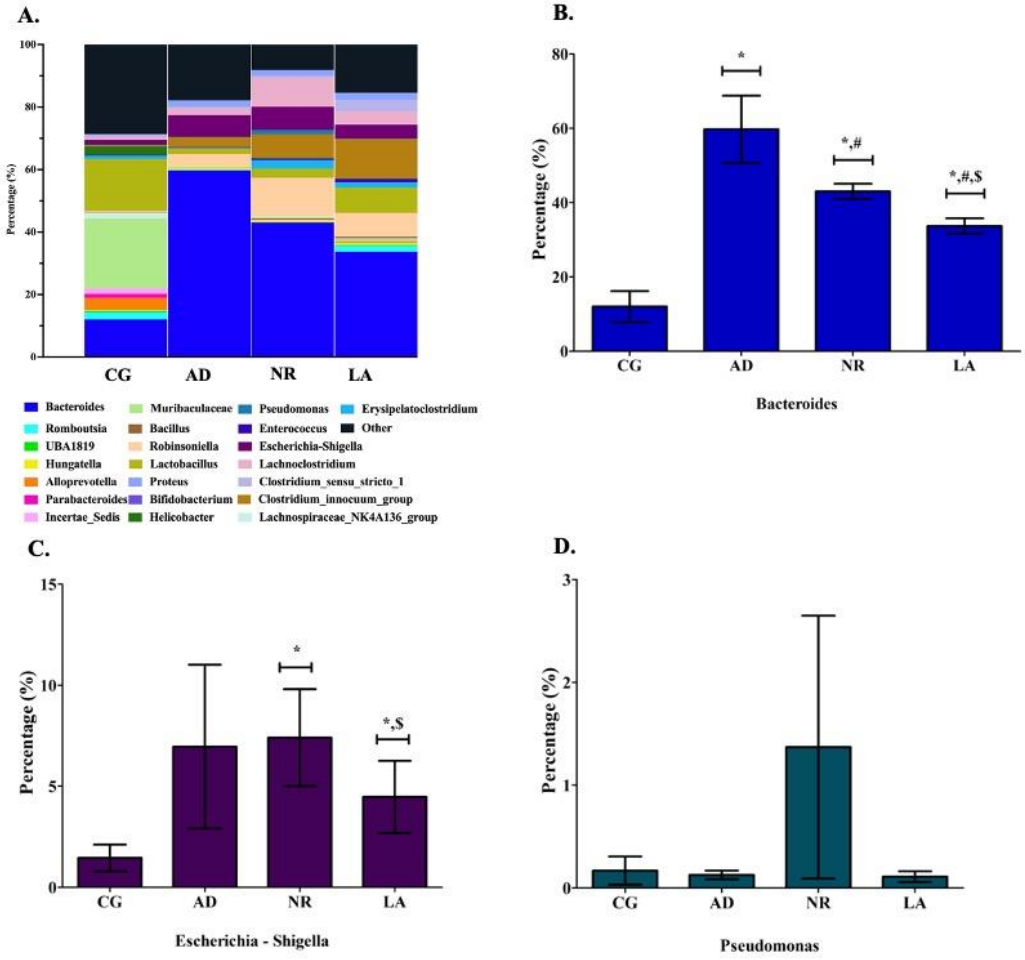

Figure-9. The genus compositions of the intestinal bacteria (A) and major differences of *Bacteroides* (B), *Escherichia Shigella* (C), *Pseudomonas* (D) of the 4 rat groups.

CG, control group; AD, AAD group; NR, natural recovery group; LA, LabMix treatment group. The Mean ± SD is used to represent the values. (*) p<0,05 compared with CG, (#) p<0.05 compared with AD, ($) p<0.05 compared with NR

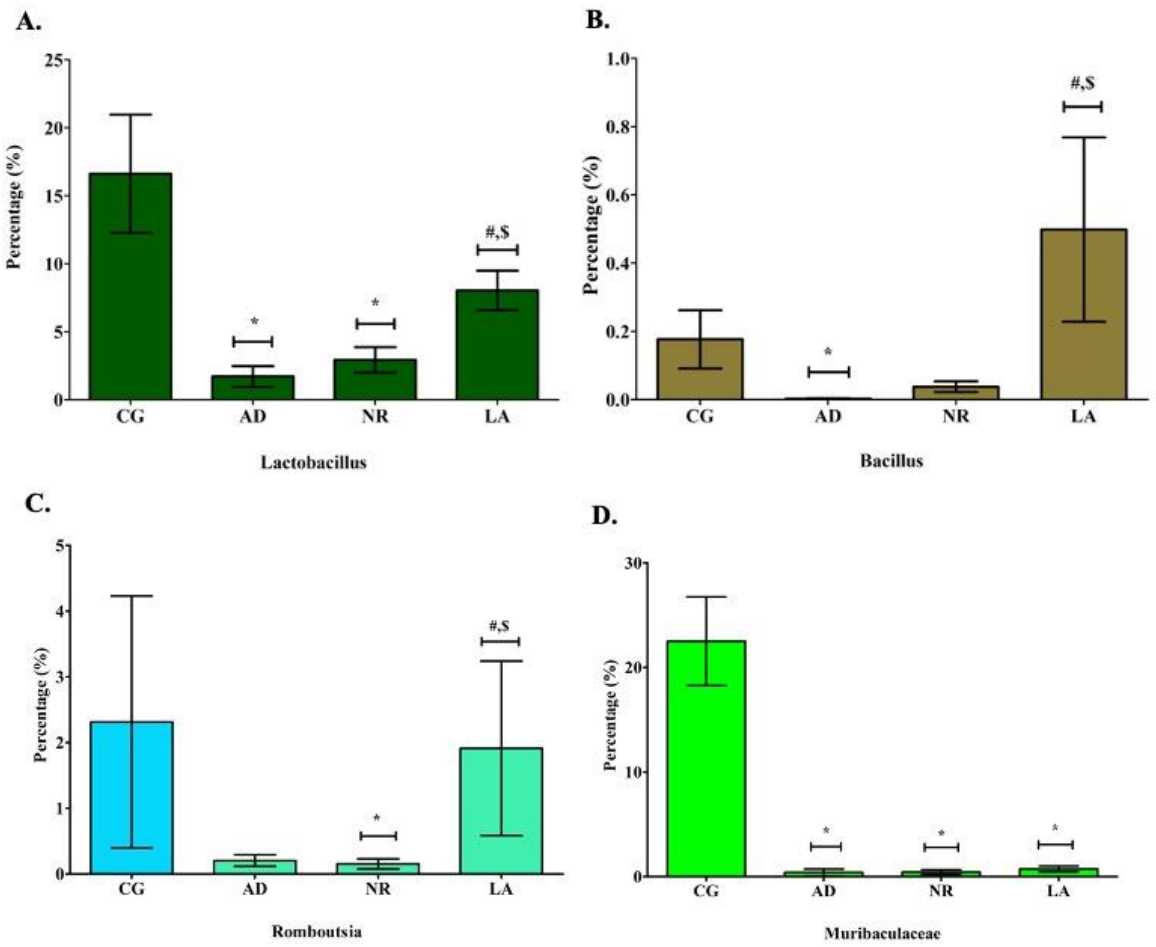

Figure-10. The main differences of *Lactobacillus* (A), *Bacillus* (B) and *Romboutsia* (C), *Muribaculaceae* (D) in the gut of the rats

CG, control group; AD, AAD group; NR, natural recovery group; LA, LabMix treatment group. The Mean ± SD is used to represent the values. (*) p<0,05 compared with CG, (#) p<0.05 compared with AD, ($) p<0.05 compared with NR

Discussion

In the LabMix preparation, the strains, including *Lactobacillus acidophilus* LA 304.17, *Lactobacillus casei* LC 304.08, and *Bifidobacterium bifidum* BF 304.98, belong to the beneficial bacterial groups Lactobacillus spp. and Biffidobacterium spp. These bacteria are commonly found in various commercial supplements for human. Both Lactobacillus and Bifidobacteria are thought to have health-promoting abilities and many of them are used as probiotics for prevention, alleviation and treatment of intestinal disorders in humans and animals. Notably, Lactobacillus and Bifidobacteria share similar biological characteristics, such as non-spore forming and the ability to grow in anaerobic condition. In addition, Lactobacillus and Bifidobacteria exhibit compatibility in the production process, including fermentation conditions, downstream processing, packing and product preservation. Therefore, we tried to combine these strains in one preparation, named LabMix, in order to assess their collective effects on the ADD rats. With this purpose we compared some indices (diarrhea severity, some immunological indices, and intestinal microbiota) between the AAD rats treated by LabMix preparation (LA group) with the untreated AAD rats, i.e., the AD and NR groups, and between the rats of the LA group with the healthy rats of the control group. The dosage of LabMix preparation was 2.52×10^9 CFU/kg/24 hours for 5 days for the AAD rats because this dose was considered as safe as the results of semi-

permanent toxicity of the LabMix preparation on the rats (the result not shown here). Additionally, probiotic characteristics directly depend on specific strains. Therefore, in this study we used the LabMix preparation with the dosage of 2.52×10^9 CFU/kg/24 hours for 5 days after 4 days of inducing diarrhea (Guo, 2021).

Antibiotics are frequently used to treat a variety of illnesses linked to inflammation and infection. Some probiotics can reduce the AAD conditions by blocking pathogens, reestablishing the proper balance of bacteria in the gut, and/or by other possible manners (Mantegazza et al., 2018). In this study, we used Lincomycin to induce AAD in the rats and then evaluated the impact of LabMix preparation on this model. Lincomycin at the dose of 5 g/kg /24 h for 4 days caused diarrhea in the rats, and diarrhea-like symptoms such as reduced body weight, nervous breakdown, dishevelled hair, and anal sticky stools appeared. Our data showed that LabMix preparation alleviated the symptoms of AAD regarding diarrhea score and cecum thickness. In particular, the diarrhea score of the LA group decreased faster than that of the NR group. In addition, the ceca in the CG and LA groups were thicker than those in the AD group ($p<0.05$), but there was no significant difference for the CG and LA groups (Figure 2B).

Antibiotic use is associated with cytokine changes which act as messengers of immune cell and indicate inflammations of the host organism. Pre-inflammatory cytokines in excess can interfere with the immune system, which can then trigger a response of inflammation. (Zhang and An, 2007). Our results showed significant increases in the levels of pre-inflammatory cytokines, including IL-6, and TNF-α, and significant decreases in the IgA concentrations in the rats' sera and intestinal mucosae in the AD and NR groups, compared with those in the CG and LA groups (Figure 4 and 5). The decrease in the IL-6 and TNF-α levels in the LA group might indicate that LabMix preparation could reduce intestinal inflammation. The results of these immune indices were consistent with the observations in the pictures of colonic tissue of the DA and NR groups that showed mild edema and inflammatory infiltrates. Our results for cytokines and IgA are consistent with other studies. Guo H. YL, 2021 showed that Bacteroides alone or in conjunction with Bifidobacterium in a mouse model of diarrhea induced by Lincomycin with the dose of 3g/kg mouse body twice a day reduced systemic inflammation, expedited tissue healing, and elevated short chain fatty acids (SCFA) levels (Guo H. YL, 2021). Furthermore, the mixed probiotic outperformed the single strain in terms of lowering colonic pathology, decreasing interleukin (IL-6) levels, and increasing the expression of the binding agent adhesion in AAD (Guo H. YL, 2021). Li et al. (2023) used ampicillin for AAD mice over three days, and then after the use of the multi-strain probiotics, including *B. lactis* XLTG11, *L. casei* Zhang, *L. plantarum* CCFM8661, and *L. rhamnosus* Probio-M9 for 14 days, the levels of cytokines IL-6, IL-1, and TNF-α reduced and the levels of cytokines IL-10 and sIgA increased (Li et al., 2023).

Probiotics have been shown in numerous studies to change the structure of gut bacteria and aid the host's microbiome to recover to normal condition following antibiotic therapy (Li et al., 2023; Yang et al., 2022). Even when the diarrhea is gone, intestinal microorganisms cannot restore to their previous state (Huse et al., 2008). In alpha diversity, the Chao 1, Simpson, Shannon and Evenness indices were the highest in the CG group and were the lowest in the NR and AD groups. In addition, the Simpson, Shannon and Evenness indices of the LA group showed significant differences compared to those of the AD group (Figure 6). The explanation for all the changes in these indices could be that the use of antibiotics decreased the diversity in the AD, NR, and LA groups compared with the CG group, and then the use of LabMix preparation could partially restore the intestinal bacteria in the LA group compared with the CG group. The PCoA revealed significant differences in the key elements of the bacterial communities in the rats' guts, which was consistent with the result of the alpha diversity analysis.

We found that not only the diversities but also the compositions of the intestinal microbiota were altered in the rats receiving Lincomycin and then LabMix preparation. Among the 3 dominant phyla, including Bacteroidota, Firmicutes, and Proteobacteria, found in rat's intestinal guts, Bacteroidota and Proteobacteria are used as indicators for some illnesses. Bacteroidota are common enteric-associated bacteria that cause diarrhea and have an inverse connection with cytokines from inflammation (Yang et al., 2021). Firmicutes are generally prevalent in the gut of healthy people and can decrease during disease, whereas Proteobacteria can be associated with a variety of chronic inflammatory intestinal illnesses (Bi et al., 2017; Kang et al., 2019). The Firmicutes/Bacteroidota ratio is often used to assess a patient's intestinal

pathologies, including IBS, IBD, and metabolic disorders (Jia et al., 2019; Louis et al., 2016). Our results showed that in the AD group, not only the Firmicutes abundance significantly reduced but also the Bacteroidota and Proteobacteria abundance significantly increased. In contrast to the AD group, in the LA group Firmicutes abundance significantly increased, while Bacteroidota and Proteobacteria abundance significantly decreased (Figure 8B, 8C, and 8D). Additionally, the ratio of Firmicutes and Bacteroidota in the LA group (0.96) was closest to that in the CG group (1.34), whereas this value in the AD group was only 0.20.

We also examined the gut microbiota at the genus level. In contrast to significant increase in the abundance of *Bacteroides* there was a significant drop in the abundance of Muribaculaceae and Lactobacillus in the AD group compared with those in the CG group. This result is consistent with those in the studies of Li et al. (Li et al., 2019), and Wang et al. (Wang et al., 2019). In comparison to the CG and LA groups, we noticed a remarkable increase in some hazardous taxa such as *Bacteroides*, *Pseudomonas*, and *Escherichia-Shigella* in the AD and NR groups (Fig. 9B, 9C and 9D). *Bacteroides* is an important genus of bacteria implicated in several gastrointestinal diseases such as *B. fragilis* is associated with clinical anaerobic infections (David Elliott and Roy, 2000), inflammatory bowel disease (Wu et al., 2004), and sepsis (Brook, 2002). In the intestinal microbiota, *Escherichia coli* and *Shigella sp*. are often the main pathogens associated with infectious diarrhea (Bona et al., 2019). In contrast to the AD and NR groups, in the LA group, the abundance percentages of these harmful bacterial genera were the lowest, but for *Bacteroides*, and *Escherichia coli* there were significant decrease ($p<0.05$) (Figure 9B, and 9C). Additionally, beneficial genera such as Lactobacillus, Bacillus and Romboutsia significantly increased in the LA group compared to those in the AD and NR groups ($p<0.05$), but there was no significant difference between the LA and CG groups ($p>0.05$) (Figure 10A, 10B and 10C). This result demonstrated that the use of LabMix preparation reduced some harmful genera and increased some beneficial genera. This also proved that LabMix preparation was able to restore partially gut microbiota in the AAD rats. Our results are consistent with those of other authors' investigations. Shi et al. (2018) conducted the study on a mouse model utilizing ampicillin for two weeks and showed the influence of a combination of four Lactobacillus species on the microbial community and the prevalence of beneficial bacteria such as Akkermansia. The use of ampicillin reduced Bacteroidetes and the addition of JUP-Y4 restored this value, which was better than spontaneous recovery. In addition, the probiotic decreased the levels of D-lactate and endotoxin in the sera, increased the expression of binding proteins, and decreased the cytokines of the intestines and colon in mice treated with antibiotics. As a result, JUP-Y4 enhanced recovery from antibiotic-induced dysbacteriosis (Shi et al., 2018). Yang et al. (2021) studied the impact of LAB-containing Lacidophilin tablets on the intestinal microbiome of AAD mice and showed that the probiotic altered substantially the structure and quantity of the bacteria in the gut. In particular, the phylum Firmicutes abundance increased while the phylum Bacteroidetes decreased (Yang et al., 2022).

Briefly, due to the use of antibiotics, healthy rats in the CG group differed from the AAD rats, including the rats in the AD, NR, and LA groups regarding some immune indices and intestinal microbiota. However, the differences between the healthy rats in the CG group and the AAD rats treated by LabMix preparation were of lesser extent than those of the AAD rats not subjected to LabMix preparation. In particular, in the LabMix treatment group, the IL-6, and TNF-α levels significantly decreased compared to those in the AD and NR groups, and these values were not significantly different between the LA and CG groups. In contrast to cytokine, in the LabMix treatment rat group, the IgA concentrations significantly increased compared to those in the AD and NR groups, but these values were significantly different between the LA and CG groups. Following the immune indices, the intestinal microbiota data of the LA group showed that the abundance of some beneficial genera increased whereas some harmful genera decreased compared to those in the untreated AAD rats, i.e., the NR and AD rat groups. In addition, some indices of the gut microbiota data of the AAD rats treated by LabMix preparation were biased, similar to those in the CG rats, and were not significantly different between the LabMix-treated and the CG rats. Moreover, significant differences in the immune indices and most intestinal microbiota data were observed between the AAD rats treated by LabMix preparation and natural recovery AAD rats. Additionally, the diarrhea score and thickness of the ceca in the AAD rats treated by LabMix preparation were better than those in the natural recovery AAD

rats which supported the results of immunological and microbiota analysis. Our results showed that the model of using LabMix preparation to mitigate diarrhea was better than the natural recovery in the AAD rats. In general, LabMix preparation showed the effects regarding the general assessments, some immune indices, and intestinal microbiota on the AAD rat model.

Conclusion

The results of this study showed the effective mitigation and the improvement of the intestinal microbiota of the AAD rats through the administration of the multi-strain probiotic preparation LabMix. The results of the general assessments (diarrhea score, and cecal thickness), specific immune indices (IL-6, TNF-α, and IgA in sera and intestinal mucosae), and amplicon metagenomic analysis (sequencing of the V3-V4 region of the 16S rDNA gene) were accordant to support that the LabMix preparation exhibited beneficial effects on the AAD rat model.

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Contribution of Authors

Nguyen DH: Performed the experiments and collected data and wrote the manuscript
Ta NAT, Van HG, Chu DT, Nguyen TS & Can VM: Analyzed and interpreted data and wrote the manuscript
Nguyen QU & Vinh HV: Conceived idea, designed and performed the experiments & collected data

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