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Research article

Evaluation of the safety and immune stimulatory effects of multi-strain Lab Mix product on laboratory animals

Duy Ha Nguyen [a], Thai Son Nguyen [a], Thi Hong Hanh Le [a], Quynh Uyen Nguyen [b], Nhat Le Bui [c,d], Dinh Toi Chu [c,d,*], Hoang Van Vinh [b,**]

[a] Vietnam Military Medical University, Hanoi, Viet Nam
[b] Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi, Viet Nam
[c] Center for Biomedicine and Community Health, International School, Vietnam National University, Hanoi, Viet Nam
[d] Faculty of Applied Sciences, International School, Vietnam National University, Hanoi, Viet Nam

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ABSTRACT

Background & aims: Probiotics are alive and beneficial bacteria used as food complements with sufficient amounts to improve and balance the intestinal flora in the human gastrointestinal tract and inhibit harmful microorganisms. In this study, we conducted experiments to evaluate he safety and the effect of one of our probiotics on selected biochemical parameters in animal models.

Methods: LabMix is a probiotic product containing three bacterial strains, including *Lactobacillus acidophilus* LA 304.17, *Lactobacillus casei* LC 304.08, and *Bifidobacterium bifidum* BF 304.98, with a density of 9×10^9 CFU/g and being mixed with suitable excipients. In this study, we conducted experiments to evaluate LabMix's acute ttoxicity in mice as well as subchronic toxicity in rats.

Results: The LD50 dose in mice of this product could not be determined since no death or disorder was recorded. In rats receiving LabMix with doses of 2.52×10^9 CFU/kg and 12.6×10^9 CFU/kg continuously for 28 days, this product caused no significant changes in the amount of red and white blood cells and platelets. Similarly, no significant changes were recorded in serum concentrations of hemoglobin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, protein, cholesterol, bilirubin, and creatinine. Besides, LabMix products also did not cause any changes in the histology of the liver, kidney, and spleen in rats. Moreover, LabMix was well tolerated without affecting the normal growth and feeding of rats. Furthermore, LabMix also decreased serum cytokines and increased serum and gut mucosal IgA antibodies.

Conclusions: LabMix product is possibly considered safe for human., and this sproduct reduced the release of pro-inflammatory cytokines (IL-6 and TNF-α), but increased IgA levels. However, it is necessary to further evaluate the product's effectiveness in the preclinical phase as well as in further phases before mass production and commercialization.

1. Introduction

Probiotics are alive and beneficial bacteria used as food complements with sufficient amounts to improve and balance the intestinal

* Corresponding author: Center for Biomedicine and Community Health, International School, Vietnam National University, Hanoi, Viet Nam
** Corresponding author: Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi, Viet Nam
E-mail addresses: toicd@vnu.edu.vn (D.T. Chu), vinhhv@vnu.edu.vn (H. Van Vinh).

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flora in the human gastrointestinal tract and inhibit harmful microorganisms, thereby enhancing health [1]. Besides, probiotics have also been studied to improve the environment such as for lead resistance, nitrite detoxification, and methane mitigation [2,3]. *Bacillus, Bifidobacterium and Lactobacillus,* and are common genera of bacteria that are commercially utilized as probiotic products. Besides, other genera like *Saccharomyces, Streptococcus, Leuconostoc, Pediococcus,* and *Enterococcus,* are also promising options for probiotic production [4]. Bacterial strains used as probiotics must be proven safe, moreover, these microorganisms must survive and grow in the host's digestive system; and inhibit microorganisms harmful to the host; thus, increasing host metabolic efficiency and immunity [1]. Lactic acid bacteria (LAB) are functional microorganisms that show their significant role in energy metabolism and optimize fermentation, especially in food fermentation due to their adaptability to acids, salts, and pH. LAB are Gram (+), oxidase (−), catalase (−), spherical or rod-shaped, non-spore-forming bacteria [4]. *Lactobacillus* spp. is a genus belonging to LAB with a large amount of Generally Recognized As Safe (GRAS) species. Their strains are commonly utilized in human nutrition and food microbiology as well as in probiotic products due to their immunosuppressive mechanisms [5]. *Lactobacillus* spp. can defeat pathogenic bacteria via their bacteriocin and other substances or at least compete with pathogens for adhesion sites and nutrition. In addition, *Lactobacillus* spp. is also widely known for its heat resistance, easy mixing, and cost-effectiveness. Although *Lactobacillus* strains are considered safe to belong to the GRAS group, several species of bacteria from this group have been reported in certain infections [6] that have shown a risk of using as a dietary supplement or biological products. *Bifidobacterium bifidum*, which are Gram (+), and are the most common bacteria in gastrointestinal tracts of birds, mammals, and some cold-blooded animals [7]. These are bacteria that appear early in the intestinal tract of infants and decrease in adulthood. They have important roles in the metabolism of mucin - an important component of the protective mucus of gastrointestinal epithelial cells. They also contribute to the evolution and maturation of the host immune system [8]. Several beneficial effects of *B. bifidum* are antibacterial properties against pathogens such as *H. pylori*, alleviating symptoms of necrotizing enterocolitis and inflammatory activity related to some chronic bowel disorders, as well as enhancing the immune system [9]. Since safety is very important for probiotic strains, various safety assessment methods such as *in vitro,* animal, and human experiments are required [10].

Three strains of *Lactobacillus acidophilus* LA 304.17*, Lactobacillus casei* LC304.08, and *Bifidobacterium bifidum* BF 304.98 that were directly isolated from Vietnamese sources are considered potential candidates for probiotics based on positive results from several *in vitro* studies. The probiotic qualities of these three strains are adherence to intestinal epithelial cells, high tolerance to acids and bile salts, and high bioactivity. Because these strains are recently isolated with no history of human consumption, evaluating the safety of LabMix products composed of these three strains is necessary. Therefore, acute toxicity and subchronic oral toxicity tests were performed as pre-clinical phases to estimate the safety of these three strains in the form of a probiotic product named LabMix. In addition, their effects on the immune system, including cytokines (IL-6, TNF-α), and antibodies (IgA) were also evaluated.

2. Materials and methods

2.1. LabMix product

LabMix is a mixture of 3 strains, including *Lactobacillus acidophilus* LA 304.17*, Lactobacillus casei* LA 304.08*, Bifidobacterium bifidum* BF304.98, in a ratio of 1:1:1, with bacterial density of 3×10^9 CFU/g for each strain. This was a product of the National Science and Technology Project, code ĐTĐL.CN-61/19 of the Ministry of Science and Technology of Vietnam. The product was manufactured according to GMP (Standards of Good Manufacturing Practice) at Nam Viet Biotechnology joint stock company. The production process, from seed selection, quality, quantity, and purity testing before freeze-drying to the manufacture of final products, was strictly controlled.

2.2. Animal

Adult Swiss mice 8–10 weeks old, healthy, meeting experimental standards, weighing 20 ± 2g were used in acute toxicity studies. Wistar rats aged 8–10 weeks, healthy, mature, weighing 200 ± 20g, and suitable for the experiment were used in subchronic toxicity studies. Study animals, provided by the Center for Experimental Animal Research - Military Medical University, were raised in laboratory conditions for 5 days before being tested. Mice and rats were fed following animal feed standards. Both studies followed the National Council's guidelines for the use and care of lab animals [11]. The experiments were conducted according to established animal welfare guidelines and were approved by an internal ethics committee of the Institute of Microbiology and Biotechnology (Decision No. 03.2021/VNU-IBMT).

2.3. Acute toxicity study

The test was performed based on guidelines of the Organization for Economic Co-operation and Development (OECD) for research on drug toxicity [12,13]. The 40 Swiss mice were randomly divided into 4 groups (10 mice/group). Before giving the drug, the mice were starved for 12 h. White mice were fed with a specially curved needle. LabMix products were mixed with distilled water with a ratio of 1:1.5 (w:v). Doses were used as follows:

Control group: Distilled water with 0.1 ml/10 g mouse body weight (BW)

Group 1: LabMix with 1.5×10^{11} CFU/kg BW.

Group 2: LabMix with 3×10^{11} CFU/kg BW.

Group 3: LabMix with 6×10^{11} CFU/kg BW.

The volume and times of oral administration were different between groups (Table 1). Each administration was 2 h apart, and the maximal administration number was 4 times per day. The interval between the highest dose that did not kill an individual mouse and the lowest dose that killed 100 % of the mice in the group was used for calculation. After the administration of Labmix, mice were fed synthetic diets provided by the Laboratory Animal Research Center while freely drinking. Follow-up was continuous for 72 h and 14 days thereafter. The ratio of dead mice was used to calculate LD50.

Clinical monitoring indicators: the number of dead mice, body weight, percentage of mice with automatic movement abnormalities (balling up in a corner of the mice cage, movement disorders), the percentage of mice showing signs of convulsions, tremors, sweating, cyanosis, and percentage of mice with abnormal changes in digestion (diarrhea).

Follow-up time: The above criteria were monitored at the following time points: Before taking the drug (T0); after 3 h (T1), after 3 days (T3), after 6 days (T6), after 9 days (T9), after 13 days (T13). Mice were anesthetized with diethyl ether and operated on, and then their livers, spleens, and kidneys were observed macroscopically and microscopically at the end of the study.

2.4. Subchronic toxicity study

Experimental design: A repeated-dose oral toxicity study lasting 28 days and following guidelines of the OECD (2008) [14]. The 30 Wistar rats were randomly divided into 3 groups (10 rats/group) and kept in the laboratory environment for 5–10 days before the experiment. Rats have received doses as follows:

Control group: Distilled water in a volume of 5 ml/kg/24.

Group 1: LabMix product with a dose of 2.52×10^9 CFU/kg (corresponding to the human dose of 2 g/50 kg/24 h)

Group 2: LabMix product with a dose of 12.6×10^9 CFU/kg (5 times higher than the human dose).

The groups of rats were orally ingested once daily in the morning for 28 days continuously.

General Observations: Observation of changes in the skin, coat, respiration, excretion of rats, monitoring of health, food intake, weight, injury, and mortality of rats throughout the experiment time.

Hematological Serum Analyze: Blood was collected into K2-EDTA tubes and automatically analyzed by Erba Elite-3 (Germany) for the count of red blood cells, white blood cells, platelets and the serum content of hemoglobin.

Biochemical Serum Analyze: Blood was collected into an appropriate anticoagulant tube and then centrifuged at 3000 rpm for 10 min to separate serum. Then the serum was automatically analyzed by AU480 - Beckman Coulter (Japan) for its concentration of AST, ALT, glucose, total protein and cholesterol, total bilirubin, and creatinine. Blood was collected with a capillary tube through the orbit at 3 time points: before the experiment (T0), after 14 days (T1), and after 28 days (T2) of LabMix preparation intake.

Histopathology: In the last step of the experiment, rats were firstly anesthetized by diethyl ether [15], then 30 % of rats in each group were dissected. Rats' kidneys, livers, and spleens were weighed, and histopathology was performed to assess the gross and microscopic damage. Visceral tissues of the kidney, liver, and spleen were fixed in 10 % formalin and then cut into 4 μm thick slices. After being stained with eosin and hematoxylin, these tissues were examined under the microscope.

2.5. Analysis of the immunological indices in blood and intestinal mucosa

Blood was taken from all groups of the rats into sterile tubes with EDTA, then centrifuged for 10 min at 4000 rpm. After removing the supernatant, the sample was stored at −80 °*C*. After preparation of the rat cecum, 100g of intestine was weighed and added into 1 ml of tissue extraction reagent I solution. The tissue sample was homogenized by Wiggens D5000 before centrifuging for 10 min at 10000 rpm, then the supernatant was aspirated for storage at −80 °*C*. To test the immunomodulatory ability of probiotic preparations, the concentration of cytokines and immunoglobulins were determined using the rat ELISA kits for IL-6, TNF-α, and Uncoated IgA (Thermo Fisher Scientific, Austria).

For cytokine index: 96 ELISA wells and chemicals were preserved at room temperature before use, with the first 7 wells for standards, the 8th well for blanks, and 50 μL of serum was added into the remaining. Another 50 μL of biotin conjugate added and incubated at room temperature (18 °*C* to 25 °*C*) for 2h. After 4 washes, each well was added 100 μl of Streptavidin HRP before being incubated at room temperature for 1h. Then continuously washing 4 times before adding 100 μL of TMB substrate and incubating for 10 min in dark condition. Finally, the ELISA product was read at 450 nm after adding 100 μL of stop solution.

For immunoglobulin: 100 μL of Coat Corning was added to 96 wells and incubated at 40 °*C* overnight. Then, add 250 μL of Blocking buffer to the wells and incubate for 2h at room temperature. The first 7 wells were reserved for the standard, the 8th well was for the blank, and 50 μL of serum was added to others. After incubation for 2h at room temperature, 100 μL of antibody detection was added and then continued incubating for 1h. After 4 times washing, 100 μL of stop solution was added after adding 100 μL of substrate

Table 1
Volume of preparations for mice.

Group (n = 10)	Vml/10g/24h	Times 1 (ml/10g)	Times 2 (ml/10g)	Times 3 (ml/10g)	Times 4 (ml/10g)	CFU/kg WB
Control	0.1	0.1	0	0	0	0
Group 1	0.25	0.25	0	0	0	$1.5x10^{11}$
Group 2	0.50	0.25	0.25	0	0	$3x10^{11}$
Group 3	1.00	0.25	0.25	0.25	0.25	$6x10^{11}$

Control group drank distilled water, group 1, 2, 3 drank LabMix preparation. Give each dose 2 h apart. Mice dose up to 4 times/day.

solution for 15 min. Results were read at 450 nm.

2.6. Statistical analysis

Data were recorded and analyzed using several software including Microsoft Excel 2013, SPSS 20.0, and GraphPad Prism 9.4.1. The mean of two standard variables was compared by paired-samples *t*-test and one-way ANOVA test. A statistically significant difference was found when p value < 0.05.

3. Results

3.1. Acute toxicity study

The acute oral toxicity study performed in rats at the doses of 1.5×10^{11}, 3×10^{11}, and 6×10^{11} CFU/kg BW did not record any death or signs of toxicity in either group. Similarly, the follow-up study of mice within 3 h–72 h and 14 days reported no movement disorders, seizures, cyanosis, dishevelled hair, or digestive disorders in all groups. Mice gained weight compared to before treatment, however, no significant difference in mouse weight among groups was detected ($p > 0.05$) (Fig. 1). No organ damage was observed at the time of surgery (Figs. 2 and 3). In general, there was no evidence of acute oral toxicity when LabMix was administered to experimental animals.

3.2. Subchronic toxicity study

General condition: The LabMix mixture consisting of 3 strains, including *Lactobacillus acidophilus* LA 304.17, *Lactobacillus casei* LC 304.08, *Bifidobacterium bifidum* BF 304.98, was orally administered at the dose of 2.52×10^9 CFU/kg BW and 12.6×10^9 CFU/kg BW for 28 consecutive days. No signs of death during the experiment, or signs of disturbances in motility, digestion, and excretion were recorded. The mean of rat body weights did not differ between the treatment groups and control group during the experimental period ($p > 0.05$) (Fig. 4).

Haematological analysis: The results of haematological parameters including the amount of red blood cells, white blood cells, hemoglobin, and platelets showed that there was no difference between control and treatment groups at T0, T1, and T2 time points during the LabMix intake (Fig. 5 A-D).

Biochemical analysis: Results of serum indices such as ALT, AST, total bilirubin, serum creatinine (Fig. 6A–D) total protein, cholesterol, and blood glucose (Fig. 7E–G) showed that there was no significant difference between control and treatment groups at T0, T1, and T2 time points of LabMix intake.

Histopathological examination: There were no abnormalities or histopathological changes in mice groups. No necrosis, fibrosis, or loss of normal structure in rat internal organs, such as the liver, kidney, spleen, or intestine, was recorded in both control and treatment groups (Fig. 8).

There were no differences in liver, kidney, and spleen weights between the control group and the treatment groups ($p > 0.05$)

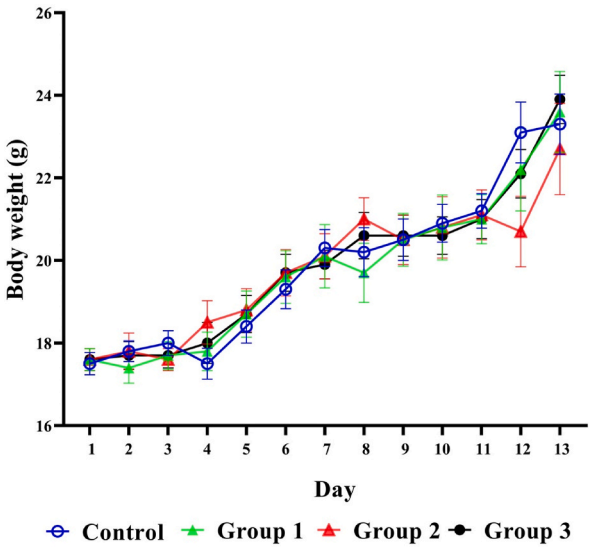

Fig. 1. Body weight of mices
Control: distilled water; Group 1: LabMix with a dose of 1.5×10^{11} CFU/kg BW; Group 2: LabMix with a dose of 3×10^{11} CFU/kg BW; Group 3: LabMix with a dose of 6×10^{11} CFU/kg BW.

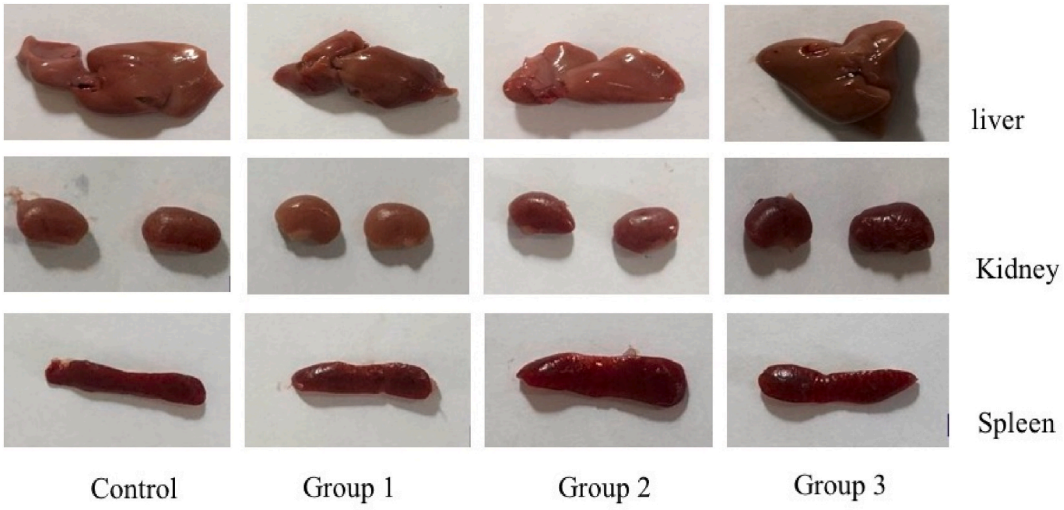

Fig. 2. General picture of the liver, kidney, spleen of mice
Control: Distilled water; Group 1: LabMix with a dose of 1.5 × 10^{11} CFU/kg BW; Group 2: LabMix with a dose of 3 × 10^{11} CFU/kg BW; Group 3: LabMix with a dose of 6 × 10^{11} CFU/kg BW.

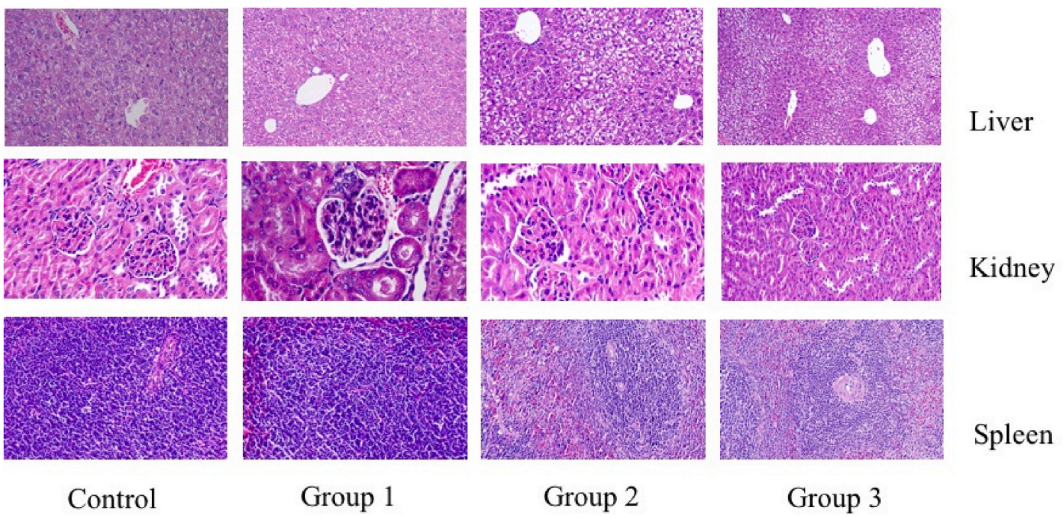

Fig. 3. Micrograph of the liver, kidney, spleen of mice
Control: Distilled water; Group 1: LabMix with a dose of 1.5 × 10^{11} CFU/kg BW; Group 2: LabMix with a dose of 3 × 10^{11} CFU/kg BW; Group 3: LabMix with a dose of 6 × 10^{11} CFU/kg BW.

(Table 2).

3.3. Analysis of the immunological indices in blood and intestinal mucosa

Cytokine index analysis: IL-6 and TNF-*α* levels in serum samples were found to decrease in the probiotic group. In particular, the IL-6 level in the control group (303.3 pg/ml) was higher than this figure for group 1 (146.65 pg/ml) and group 2 (154.42 pg/ml) with a statistically significant difference ($p < 0.05$). Similarly, the TNF-*α* level in the control group (356.9 pg/ml) was higher than that in group 1 (191.7 pg/ml) and group 2 (232.29 pg/ml) with $p < 0.05$ (Fig. 9 A, B).

Immunoglobulin index analysis: Serum IgA and secretory IgA levels of the intestinal mucosa both increased in the probiotic group compared to the control group ($p < 0.05$) (Fig. 9C, D). The serum IgA level in the probiotic group and the control group was 5.99 ng/ml, 6.44 ng/ml, and 3.02 ng/ml, respectively, while figures for the IgA level in the intestinal mucosa were 0.33 ng/ml, 0.36 ng/ml and 0.11 ng/ml, respectively.

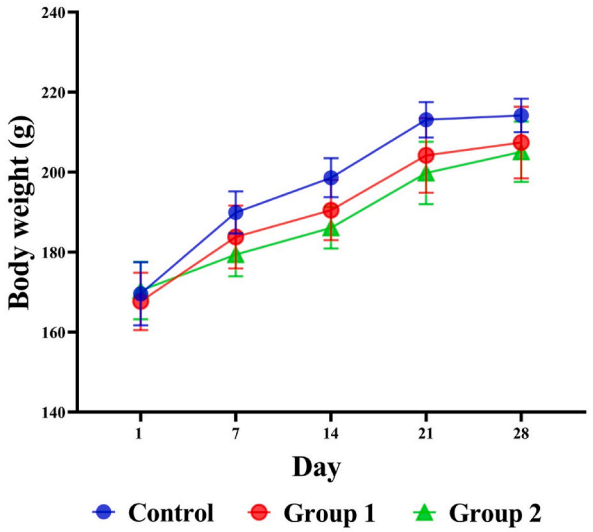

Fig. 4. Body weight of rats
Control: Distilled water; Group 1: LabMix with a dose of 2.52 × 10^9 CFU/kg BW; Group 2: LabMix with a dose of 12.6 × 10^9 CFU/kg BW.

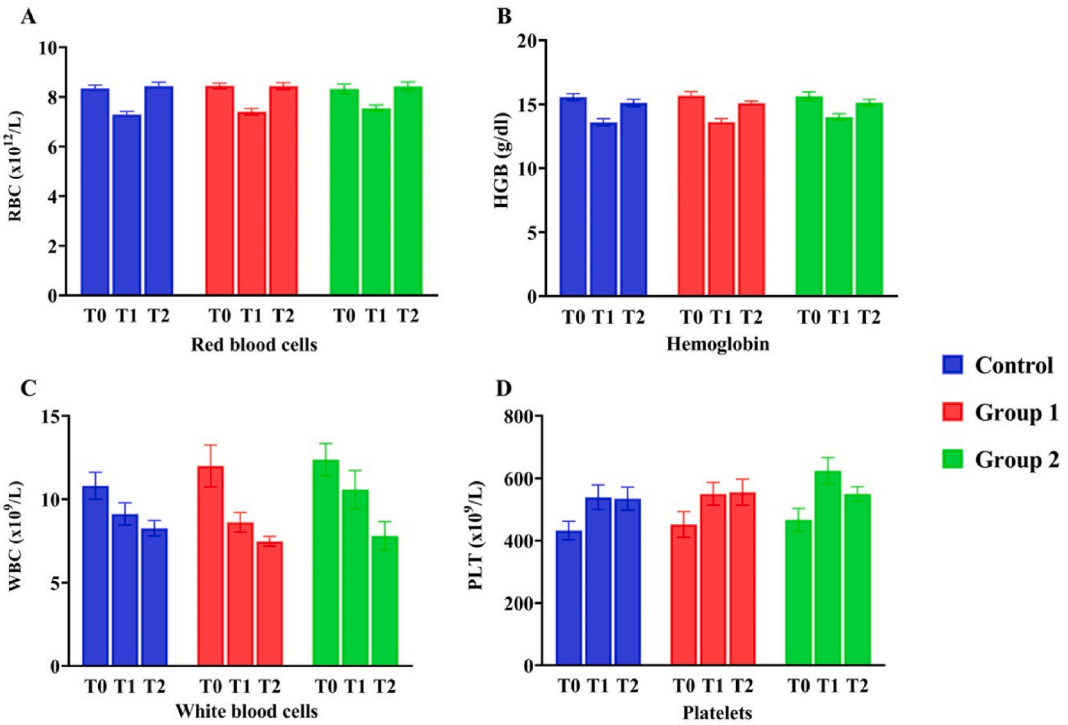

Fig. 5. Hematological parameters of rats
Control: Distilled water; Group 1: LabMix with a dose of 2.52 × 10^9 CFU/kg BW; Group 2: LabMix with a dose of 12.6 × 10^9 CFU/kg BW
A: Red blood cells (RBC), B: Hemoglobin (HGB), C: White blood cells (WBC), D: Platelets (PLT), T0: before treatment, T1: after 14 days, T2: after 28 days.
(For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Although *Lactobacillus* strains are considered safe for probiotic products since they belong to the GRAS group, WHO/FAO (2002) still recommended that the safety of these strains should be evaluated before use in humans to make sure of their harmlessness to the host [10]. Currently, there are very few reviews of the safety and efficacy of multi-strain probiotics worldwide, primarily reviews of

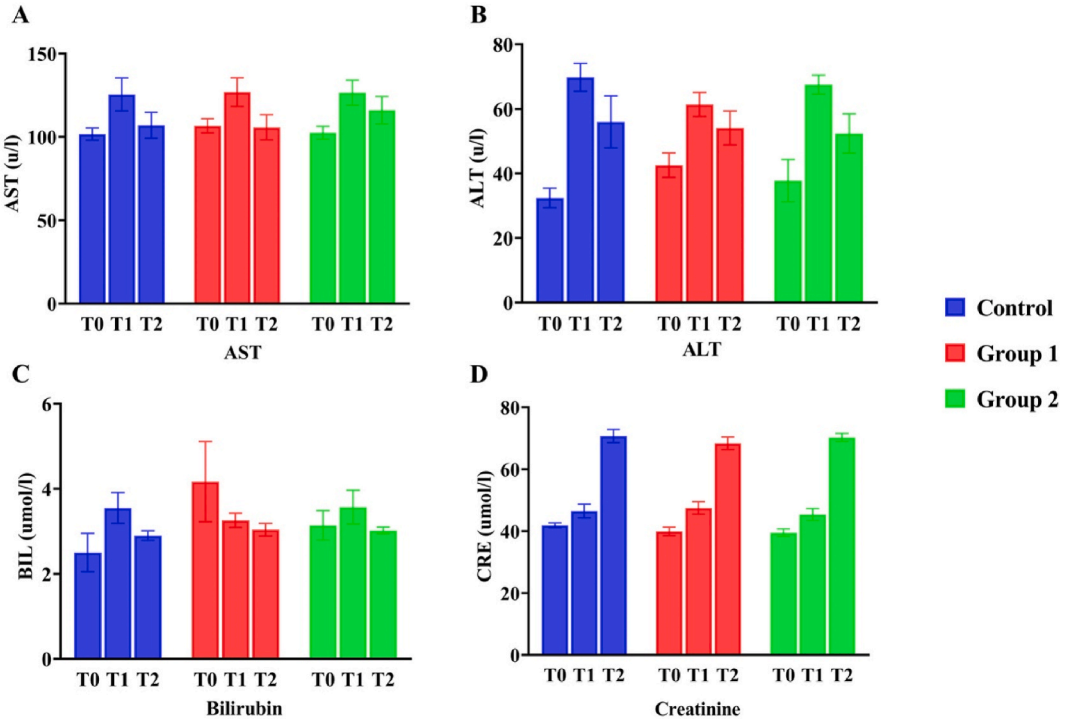

Fig. 6. Biochemical parameters of rats
Control: Distilled water; Group 1: LabMix with a dose of 2.52 × 10^9 CFU/kg BW; Group 2: LabMix with a dose of 12.6 × 10^9 CFU/kg BW
A: AST, B: ALT, C: Bilirubin (BIL); D: Creatinine (CRE); *T0: before treatment, T1: after 14 days, T2: after 28 days.*

single-strain probiotics. The advantage of LabMix is that it is a multi-strain preparation consisting of 2 *Lactobacillus* strains and 1 *Bifidobacterium* strain. The safety profile of each strain has been studied and confirmed. Safety has been demonstrated for other *L. casei* strains in various animal models. Male BALB rats were orally dosed with *L. casei* IMVB-7280 at 5 × 10^6, 5 × 10^8, 5 × 10^9 CFU/day for 7 days or 1 × 10^{10} CFU/day for 3 days, and a follow-up of 14 days. The survival rate of all mice was 100 %, and the weight remained unchanged [16]. The strain L. *acidophilus* was tested for its function against 1,2-dimethylhydrazine (DMH) and sodium dextran sulfate (DSS)-induced colon cancer in mice. Results showed L. *acidophilus* helped in preventing colorectal precancerous lesions [17]. The animal safety assessment is an important step before a product can be used for humans. In this study, the safety of the LabMix product was evaluated by acute toxicity and subchronic oral toxicity tests. Mice were used to experiment due to the advantage of the mice model that the mice have the same physiological conditions as humans and are easy to guide [18]. The experimental conditions strictly followed the guidelines of the OECD/OCDE 2008, and the national guidelines for the care and use of laboratory animals.

The 14-day acute oral toxicity results were used to calculate the LD50 dose (the dose that kills 50 % of the test animals) to derive the dose to be used in the human and subchronic toxicity study. The results of the acute oral toxicity test reported no death or signs of abnormalities in movement with 100 % of mice walking normally. No disorders such as cramps, tremors, increased sweating, or cyanosis; and no digestive disorders such as decreased appetite, diarrhea, etc. were found in experimental groups. Therefore, the LD50 dose of the rennet preparation in white mice could not be determined, even at the dose of 6 × 10^{11} CFU/kg BW, which corresponds to a dose of 68 g/kg BW of LabMix (139 times higher than the dose for human). The acute oral toxicity of various *Lactobacillus* and *Bifidobacterium* strains has been studied [19,15]. Studies have shown that no signs of acute toxicity were observed after using the doses of 1.7–4.1 × 10^{12} CFU/kg body weight (100 times higher than the dose used).

Since the acute toxicity data were not sufficient for the safety of the product, we performed a subchronic toxicity test on rats, orally administered at doses of 2.52 × 10^9 CFU/kg BW (equivalent to the human dose) and 12.6 × 10^9 CFU/kg BW (5 times higher than human dose) for 28 days. The results of the subchronic toxicity test reported no abnormalities in rats even at the high dose of 12.6 × 10^9 CFU/kg BW (the recommended dose of almost all probiotics for humans range from 10^8 to 10^9 CFU/kg BW). Mouse weight is one of the most important indicators to assess the normal growth of a mouse. In this study, the weight of rats did not change between the control and treatment groups for 28 days, while it steadily increased after the experiment. Thus, it could be concluded that LabMix did not affect the normal growth of rats. After dissecting the rat, organ weights were measured to evaluate changes in some internal rat organs such as the spleen, kidney, and liver. The result showed that no significant changes were found between groups. In the macroscopic and microscopic pathology, no damage in the rat's internal organs such as the spleen, kidney, liver, and intestine was observed. The production did not affect the normal structure of the organs, and no abnormalities or lesions were found. Our study showed that there was no difference between the treatment and control groups in terms of general conditions such as body weight, movement, excretion, gait, skin, hair; hematological, biochemical, and pathological indicators in the 28-day trial. The study by Owaga

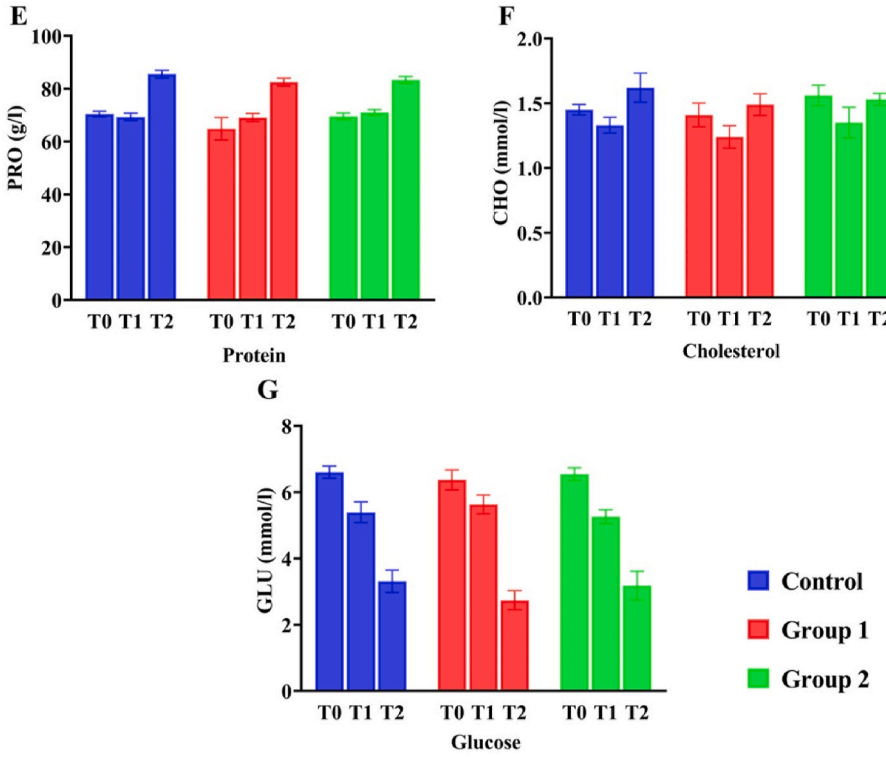

Fig. 7. Biochemical parameters of rats
Control: Distilled water; Group 1: LabMix with a dose of 2.52 × 10^9 CFU/kg BW; Group 2: LabMix with a dose of 12.6 × 10^9 CFU/kg BW
E: Protein (PRO), F: Cholesterol (CHO), G: Glucose (GLU); T0: before treatment, T1: after 14 days, T2: after 28 days.

et al. evaluating the safety of *Lactobacillus kefiranofaciens* M1 in rats over 28 days at the doses of 3 × 10^8, 9 × 10^9, 1.8 × 10^{10} CFU/kg body weight showed that different doses did not affect the growth of rats [19]. Shokryazdan et al. investigated a combination of *L. buchneri* FD2 and *L. fermentum* HM3 strains in rats at two different doses (10^9 and 10^{10} CFU/kg body weight) and indicated that probiotic products did not affect biochemical and hematological as well as biochemical parameters in rats [15]. Evaluation of acute toxicity and chronic toxicity in 90 days of the probiotic product Lab4, composed of 2 strains of *Lactobacillus* and 2 strains of *Bifidobacterium,* showed the product was safe at the dose of 5 × 10^{11} CFU/kg/day [20].

Another concern with probiotic strains is the potential for immunogenicity when used. Here we examined the cytokine index IL-6 and TNF-α. These two cytokines play important roles in some immune functions and metabolic disorders. According to a study testing and examining several probiotic strains by Murat Karamese et al., all tested strains reduced IL-6 and TNF-α levels [21]. This result was consistent with the report of Parisa Shokryazdan et al. using *Lactobacillus* strains [15]. However, the underlying mechanism of immune regulation of probiotics through cytokines is currently unclear. The differences in the results of these studies could be explained by differences in selected strains. Our study showed a remarkable increase in IgA levels in the probiotic group. IgA is mainly produced in the intestinal mucosa and plays an important factor in the body's natural immunity. The increase of IgA levels in serum and gut mucosa could also be explained by increased production of short-chain fatty acids related to gut microbiota.

Regarding probiotics' effect on the environment, we supposed that probiotic products are completely natural beneficial bacteria, these beneficial bacteria are all isolated from fermented foods, or the human intestinal tract, so they are quite safe and environmentally friendly. Probiotics are healthy foods for humans and animals because they enhance the host's resistance, reducing the host's risk of disease. Thereby reducing the proliferation and spread of harmful bacteria into the environment from humans and especially from livestock.

One limitation of this study was that the effect of the product was not evaluated in different breeds of studied rats. However, the preparation has been particularly assessed by recent evaluating methods to evaluate the safety of probiotics before use for humans [22]. The potential results of these investigations may assure further advances for carrying out further clinical studies [23]. Potential use of a multi-strain preparation including strains of *Lactobacillus* spp. and *Bifidobacterium* spp. for viral pathogens, limits measures to use toxic chemicals to kill viruses [24]. Encouraging research into safety issues and prescribing for patients using probiotics in treatment. This aspect prompts further studies of longer duration (60 days, 90 days) and in different animal models with different diseases.

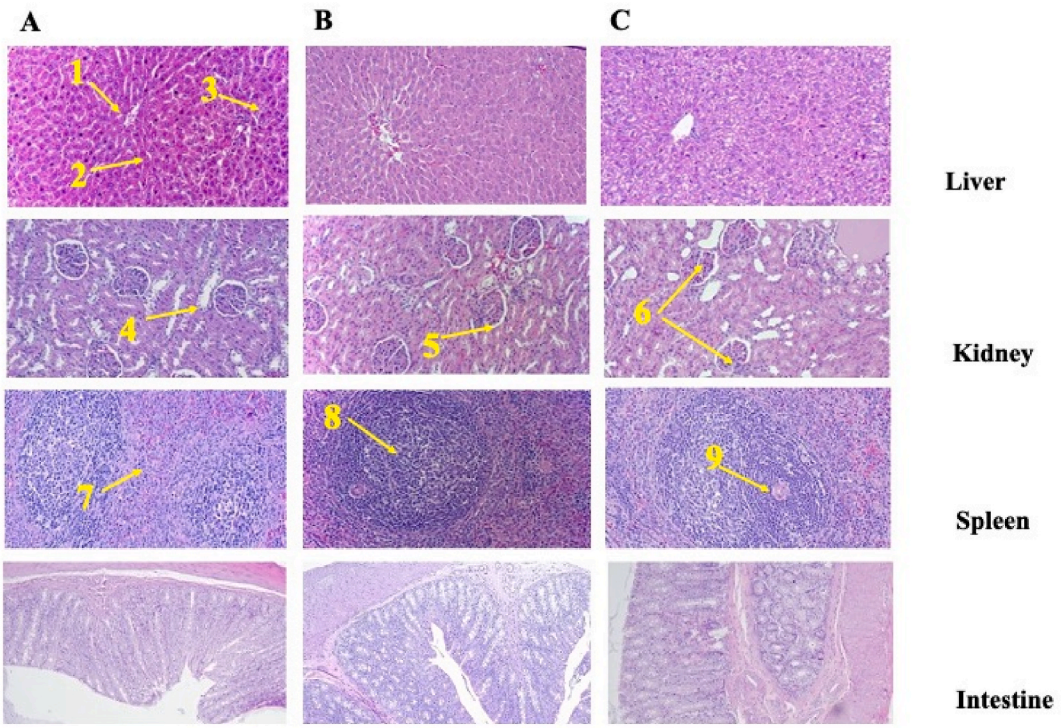

Fig. 8. Histopathological picture of the liver, kidney, spleen and intestine of rats.
A: distilled water, B: distilled water +2.52 × 10^9 CFU/kg BW of LabMix, C: distilled water +12.6 × 10^9 CFU/kg BW of LabMix
1: central vein; 2: sinusoids; 3: sheets of hepatocytes, 4: renal tubules; 5: Bowman's capsule; 6: glomerulus, 7: red pulp; 8: white pulp; 9: lymphoid follicles.
Liver:.Hepatocytes were arranged in bands, raft walls, and between bands, the liver rafts have vascular sinuses. Liver cells were not broken down. The sinuses were easily congested.
Kidney: *The renal cortex had glomerulus, tubules, and blood vessels between the tubules. Renal tubular epithelial cells did not degenerate. The blood vessels were easily clogged.*
Spleen: Splenic parenchyma with white and red pulp. The white pulp region had fairly uniform lymphoid follicles with a central keel artery. The red pulp region contained the Billroth cord and vascular sinuses.
Intestine: *The intestinal mucosa was thick with superficial papillary and ductal glands. Epithelial cells had small and regular nuclei. The stroma of the capillaries was slightly obstructed. There were cells of the lymphatic system.* (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Weight of rat organs[#].

	Liver	Kidney	Spleen
Control	6.43 ± 1.84	1.23 ± 0.15	0.55 ± 0.12
Group 1	6.38 ± 1.13	1.30 ± 0.11	0.60 ± 0.13
Group 2	6.34 ± 1.19	1.25 ± 0.10	0.57 ± 0.10

[#]Mean standard deviation of 10 replicates.
Control group: distilled water, Group 1: 2.52 × 10^9 CFU/kg BW of LabMix, Group 2: 12.6 × 10^9 CFU/kg BW of LabMix.

5. Conclusions

In this study, we performed acute toxicity and subchronic toxicity oral tests to assess the safety of LabMix - a probiotic product containing *L. casei, L. acidophilus,* and *B. bifidum* on laboratory animals. No abnormalities in general assessment and biochemical as well as hematological parameters were observed in treatment groups. Since the LD50 dose of the product in laboratory animals could not be determined, LabMix is considered safe to use for humans. LabMix reduces the release of pro-inflammatory cytokines like IL-6 and TNF-α, which can act as anti-inflammatory supplements. Besides, LabMix increased IgA levels, which enhances the natural immune system. However, it is necessary to further evaluate the product's effectiveness in the preclinical phase as well as in further phases before mass production and commercialization.

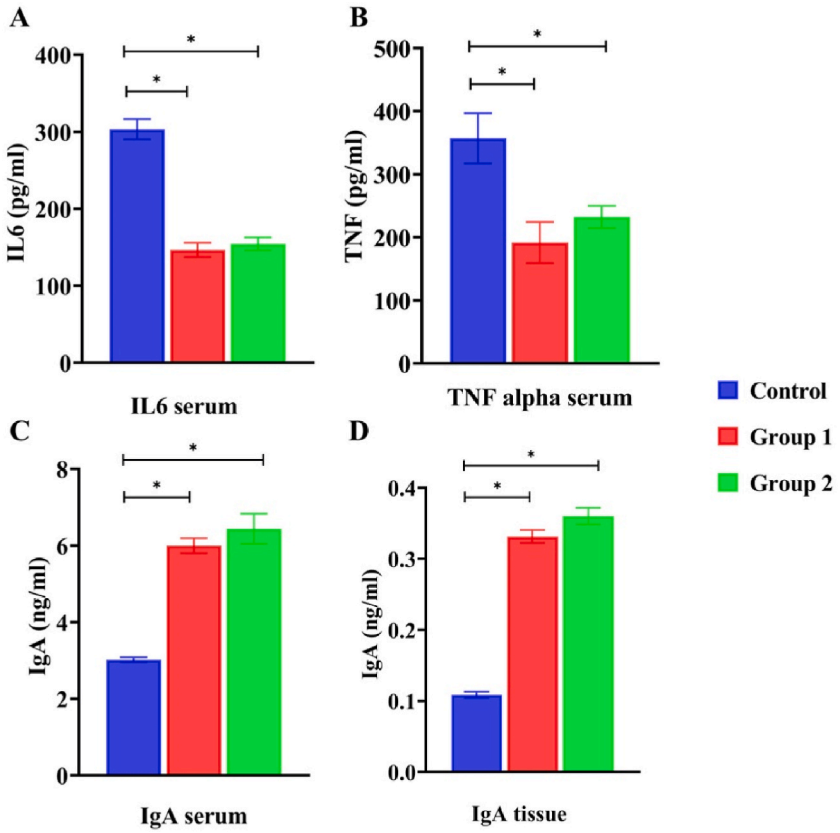

Fig. 9. Cytokine and immunoglobulin of rats
Control group: distilled water, Group 1: 2.52 × 10^9 CFU/kg BW of LabMix, Grorp 2:12.6 × 10^9 CFU/kg BW of LabMix
A: IL 6, B: TNF-α; C: IgA serum; D: IgA tissue, *p < 0.05.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Duy Ha Nguyen: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Thai Son Nguyen:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Thi Hong Hanh Le:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Quynh Uyen Nguyen:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Nhat Le Bui:** Writing – review & editing, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Dinh Toi Chu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Hoang Van Vinh:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

ALT Alanine aminotransferase
AST Aspartate aminotransferase

BW	Body weight
CFU	Colony Forming Unit
GRAS	Generally Recognized as Safe
LAB	Lactic acid bacteria
OECD	Organization for Economic Cooperation and Development

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