

Safety assessment and compatibility of multi-strain probiotic *Lactiplantibacillus plantarum* subsp. *plantarum* (strain Dad-13 and FNCC-0250) and *Lacticaseibacillus paracasei* GMRMP-001 in vivo

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Abstract

Lactiplantibacillus plantarum subsp. *plantarum* Dad-13, *L. plantarum* subsp. *plantarum* FNCC 0250, and *Lacticaseibacillus paracasei* GMRMP-001 are isolated from Indonesian traditional fermented food, and their combination may function as a probiotic. This study aimed to evaluate the compatibility and safety assessment of the multi-strain probiotic. In this research, organ weight, hematological parameters, SGOT, SGPT, translocation of bacteria in the blood and organs were evaluated, and 16S rRNA gene sequencing was performed to confirm the identity of the strain. The study outcomes indicated that administering a repeated dose of the P4 group did not lead to any toxic effects in rats, even at a high concentration, it can be concluded that GMRMP-001 at high doses did not cause bacterial translocation into the blood and other organs of the rats. The average populations of *L. plantarum* and *L. paracasei* in the multi-strain probiotic group showed compatibility with multi-strain probiotic.

1. Introduction

Probiotics are living microorganisms that can provide health benefits to their hosts if consumed in adequate amounts (FAO/WHO, 2002). Multi-strain probiotic offers more significant advantages than single-strain due to the synergistic effects of combining various isolates (Kwoji *et al.*, 2021). *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13 (Dad-13) and *L. plantarum* subsp. *plantarum* FNCC-0250 (FNCC-0250) are two local probiotics which have been proven safe for consumption (Rahayu *et al.*, 2019; Ikhsani *et al.*, 2020). *Lacticaseibacillus paracasei* GMRMP-001 (GMRMP-001) is a probiotic candidate isolate from Dadih in Alahan Panjang, Indonesia. Based on the different origins and characteristics of these three local probiotic strains, researchers aimed to combine local strains of the bacteria Dad-13, FNCC-0250, and GMRMP-001 to evaluate the compatibility of the strains and prove their safety before applying them to commercial.

A previous study done by (Rahayu *et al.*, 2019; Ikhsani *et al.*, 2020) reported that Dad-13 and FNCC-0250 could not translocate to the organs or blood of the rats. Rossi *et al.* (2022) and Kullar *et al.* (2023) reported that the occurrences of sepsis, and bacteremia linked to

the translocation of bacteria. A safety evaluation of GMRMP-001 must be done since probiotic bacteria's characteristics vary depending on the strain (Salminen *et al.*, 1998).

Certain species of bacteria, when combined as a formulation, can inhibit other species. (Agyeman *et al.*, 2017). Therefore, it is essential to carry out research regarding the compatibility and safety of multi-strain probiotics (Buiatte *et al.*, 2023). The safety of the probiotic was assessed through various evaluations involving rats. These included evaluating their feed consumption, tracking changes in body and organ weight index of rats, changes in the intestinal of rats, blood biochemical tests such as serum glutamic oxaloacetic (SGOT) and glutamic pyruvic transaminase (SGPT), gut histological analysis, and bacterial analysis to detect translocation in the blood and organs such as bacterial genome DNA isolation, repetitive-polymerase chain reaction amplification, 16S rRNA gene amplification and sequencing analysis-the absence of significant effects of multi-strain probiotic treatment led to its confirmation as safe for consumption.

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2. Materials and methods

2.1 Bacterial strain and cell production

Bacterial strains used in this study included *Lactiplantibacillus plantarum* subsp. *plantarum* Dad-13, *L. plantarum* subsp. *plantarum* FNCC 0250, and *Lacticaseibacillus paracasei* GMRMP-001. These were sourced from the Food and Nutrition Culture Collection, Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. Single-strain and multi-strain probiotic production was done following A'inurrofiqin et al. (2022) with modification. Each strain was inoculated into 5 mL de Man Rogosa Sharpe (MRS) broth to produce biomass for multi-strain probiotics and incubated at 37°C for 24 h. After inoculation, the cultures were transferred into 100 mL of MRS broth, incubated at 37°C for 24 h, then centrifuged. The resulting pellets were mixed and dissolved in 200 mL of 10% skim milk solution at a concentration of 10⁹CFU/mL.

Production of biomass single-strain probiotic was carried by inoculating *L. plantarum* subsp. *plantarum* Dad-13 into 5 mL of de Man Rogosa Sharpe (MRS) broth. Then, it was inoculated (approximately 2 mL) into 200 mL of MRS broth and incubated 24 h, 37°C. The culture was centrifuged, and the pellet was dissolved in 200 mL of 10% skim milk solution at a concentration of 10⁹CFU/mL.

2.2 Experimental design

This study used Sprague–Dawley rats as the research subjects. The research had been approved by the Ethical Clearance Committee, Integrated Research and Testing Laboratory (IRTL), Universitas Gadjah Mada (UGM), Indonesia, with the ethical clearance certificate numbered 00046/04/LPPT/II/2023. Twenty-four male rats (8 weeks; 200±20 g) were used in this experiment. The rats were fed with AIN-93M and provided water *ad libitum* based on the method (Reeves et al., 1993). The rats were divided into four groups (P1, P2, P3, and P4; n = 6). P1 was the control group. P2 was given 1 mL of 10% skim milk. P3 was given 1 mL of 10⁹CFU *L. plantarum* subsp. *plantarum* Dad-13 in 10% skim milk. P4 was given 1 mL of 10⁹CFU multi-strain probiotic in 10% skim milk. All rats were euthanized with ketamine on day 29. After that, blood and organs were analyzed.

2.3 General health, hematological, and biochemical analyses

The weights of organs including kidney, spleen, and liver were also recorded. The hematological parameters were analyzed using a KX-21 Hematology Analyzer (Sysmex, Japan). Additionally, the SGOT and SGPT levels were measured using a DyaSis kit (Holzheim,

Germany). An organ weight index was measured based on a formula according to Zhou et al. (2000).

2.4 Gut histological analysis

The tissue samples including ileum, cecum, and colon were fixed using 10% formalin and molded in paraffin blocks and cut into 3–4 µm lengths. The tissue blocks were stained with Hematoxylin and Eosin solution following Zhou et al. (2000).

2.5 Bacterial analyses of digesta, feces, blood, and organ

Approximately 0.5 g each of digesta and feces samples were serially diluted with 4.5 mL of a 0.85% NaCl. Subsequently, each sample was inoculated in MRS agar, *Lacticaseibacillus paracasei* Selective Media (LLV) and *Lactiplantibacillus plantarum* Selective Media (LPSM). The samples were incubated at 37°C for 48 - 96 h. The formulations of LPSM and LLV followed the method described by Yuki et al. (1999) and Bujalance et al. (2006), with modifications.

Each organ and blood were enriched in MRS broth and incubated at 37°C for 24 h. Subsequently, the enriched sample was streak-inoculated on LLV media and incubated. Further analysis included cell morphology, Gram staining, and catalase following the method by Zhou et al. (2000) with some modifications.

2.6 Bacterial genome DNA isolation

All bacterial colonies from blood and organs in LLV media, with the same appearance as *Lacticaseibacillus paracasei* were collected and cultivated in MRS broth. After the bacteria were cultivated, DNA was extracted and purified to confirm the identity following the method of Serra et al. (2018) and Geneaid Biotech (2022). Bacterial genomic DNA was isolated from the rats' blood, organs, and colon using the Presto™ Mini gDNA Bacteria Kit (Geneaid, New Taipei City, Taiwan) based on the manufacturer's protocol. (1) Sample preparation: The bacterial cells were transferred to microcentrifuge tube, which was centrifuged, and the supernatant was discarded. An aliquot of the Gram + Buffer was transferred per sample to centrifuge tube, Lysozyme (0.8 mg/200 µL) was added and vortexed to fully dissolve the Lysozyme. The pellet was resuspended and Gram + Buffer was added to the sample in the microcentrifuge tube and incubated for 30 min at 37°C. The tube was inverted every 10 min during the incubation. Proteinase K was added, vortexed, and incubated for at least 10 min at 60°C. The tube was inverted every 3 min during the incubation; (2) Lysis: A 200 µL aliquot of GB Buffer was transferred to the sample, vortex mixed, and incubated for at least 10 min at 70 °C to make sure the sample lysed. The tube was

inverted every 3 min throughout the incubation. The Elution Buffer was pre-heated to 70°C; (3) DNA binding: Transfer absolute ethanol to the sample lysate and shake the mixture. The mixture was transferred to a GD column with a 2 mL collection tube. Centrifuge the mixture and remove the collection tube holding the flow-through. Add a new collection tube to the GD column ; (4) Wash: Transfer W1 Buffer to the GD column and centrifuge; remove the flow-through, put the GD column back on the collection tube, transfer Wash Buffer (ensure ethanol was added) to the GD column, centrifuge; remove the flow-through, put the GD column back with the collection tube and centrifuge again to dry the column matrix; (5) Elution: The dried GD column was filled and attached to a clean microcentrifuge tube; pre-heated Elution Buffer was transferred into the center of the column matrix, ensure that the Elution Buffer was fully absorbed then centrifuged to elute the purified DNA. Finally the isolated DNA was stored at -20°C.

2.7 Repetitive-polymerase chain reaction amplification

The PCR-mix Ready to Go (GE Healthcare, Germany) was used for the PCR amplification. Each ampoule PCR-mix reaction was mixed with DNA, BOXA1R primer (5'CTACGGCAAGGCAAGGCGACGCTGACGCTGACGCTGACG-3'), and nuclease-free water. The PCR amplification procedures followed the method of Ikhsani *et al.* (2020), where the initial denaturation (94°C for 4 min), 30 cycles of denaturation at 92°C for 1 min, annealing at 50°C for 1 min, extension at 68°C for 8 min, and the final cycle at 65°C for 10 min. PCR products were analyzed using ethidium bromide staining on a 2% agarose gel submerged in 0.5× TBE at 100 V for 30 min. Bacterial isolates were matched with the reference DNA of *Lactocaseibacillus paracasei* GMRMP-001 to generate fingerprints, which were used to create dendrogram similarities (Ikhsani *et al.*, 2020).

2.8 16S rRNA gene amplification and sequencing analysis

16S rRNA gene amplification was performed following A'inurrofiqin *et al.* (2022) method. One µL of DNA, 1 µL each of the 27F and 1492R primers, and 22 µL of nuclease-free water were added to each PCR-mix RTG ampoule. There were multiple phases involved in the PCR amplification process using the Thermal Cycler TC1000-G (DLAB Scientific): (1) Initial denaturation (96°C for 4 min) in one cycle, (2) 35 cycles of denaturation (94°C for 1 min), annealing (52°C for 1.5 min) and extension (65°C for 8 min), (3) one final cycle (68°C for 10 min) and (4) cooling (12°C for 10 min), followed by electrophoresis of the DNA fragments. The DNA sequencing was conducted in IRTL UGM.

ExoSap_IT, Bigdye Terminator kit (Cycle sequencing), and Bigdye-X-Terminator purification were used for General Sequencing and purification. The results of the DNA sequences were read using DNA Baser software.

2.9 Statistical analysis

The statistical significance was assessed using one-way analysis of variance (ANOVA), Kruskal Wallis, and Mann-Whitney. A significant difference was set when p Values < 0.05.

3. Results and discussion

3.1 Health indicators, hematological and biochemical parameters

All rats in groups P1, P2, P3, and P4 survived and showed no significant changes in body weight until the last day of the experiment. Overall, groups P1, P2, P3, and P4 from weeks 0-4 showed no significant decrease in feed intake, which indicated consistency in the physiological conditions of rats. A decrease in feed intake is a symptom of health issues, including stress or infection (Sominsky and Spencer, 2014).

Changes in the weight ratio of a specific organ are an indicator of infection. The organ weight index per group is displayed in Table 1. The selection of the kidney, spleen, and liver to be observed was because these organs are representative organs that react to toxicity (Merouani *et al.*, 2017). The kidney weight index was not different in all groups ($p > 0.05$); the spleen weight index between the control and multi-strain group also showed no significant difference, but there was a significant difference between P1 and P4 in the liver weight index. Although there was a significant difference in liver weight index, in the multi-strain probiotic group, there was no enlarged effect compared with control, which indicates that multi-strain did not cause liver inflammation.

Table 1. Organ weight index.

Group	Organ (g)		
	Kidneys	Spleen	Liver
P1	0.99±0.17 ^a	0.36±0.06 ^{ab}	5.08±0.50 ^{bc}
P2	1.02±0.08 ^a	0.46±0.06 ^{ab}	5.44±0.34 ^c
P3	0.88±0.79 ^a	0.35±0.07 ^a	4.56±0.60 ^{ab}
P4	0.95±0.08 ^a	0.48±0.17 ^b	4.26±0.62 ^a

Values are presented as mean±SD. Values with different superscripts in the same column are statistically significantly different ($p < 0.05$).

Hematological parameters are used to evaluate the harmful effects of test substances on blood (Zhou *et al.*, 2000). There were no significant differences in red blood cells and leukocytes, except lymphocytes and

neutrophils. It shows that there is no infection caused by the treatment. The neutrophils were higher in the P4 group compared to control. However, according to Alemán *et al.* (1998), the normal range of neutrophils in rat blood was 12.75-41.95%. This result shows that a high dose of multi-strain did not cause infection in the blood.

An increase in SGOT and SGPT can be caused by damage to body cells or infection (Amala *et al.*, 2016). The levels of SGOT and SGPT are displayed in Table 2. Consumption of a high dose of multi-strain probiotics showed no significant difference in SGPT in all groups. SGOT level was significantly different between all groups. However, the highest SGOT level was measured in the control group; it is suggested that the administration of GMRMP-001 and the multistrain group did not induce the SGOT and SGPT levels.

3.2 Gut histology analysis

As can be seen in Figure 1, there were no notable alterations to the gut morphology. The epithelial was intact, there was no alteration of the shape of villi among all groups, no widening of space between villi. Small intestinal inflammation is indicated by a decrease in small intestine length, and a shortening of the villi (Kühnel, 2003; Kato *et al.*, 2017). Translocation is thought to be a sign of impaired intestinal integrity. The leakage between the intestinal epithelial cells (IECs) would allow bacteria pass through the intact gut epithelial layer (Nagpal and Yadav 2017; Mayangsari, Okudaira, Mano *et al.*, 2021; Mayangsari, Sugimachi and Xu *et al.*, 2021). In this study, the villous and epithelial height of the ileum, caecum, and colon of the multi-strain group did not differ from the control, skim milk, and Dad-13 group. This can be seen in Table 3.

Table 2. Blood hematology result.

Parameter	P1	P2	P3	P4
WBC ($10^3/\mu\text{L}$)	18.88±4.2 ^a	18.95±6.44 ^a	14.36±2.90 ^a	17.58±3.37 ^a
RBC ($\times 10^6/\mu\text{l}$)	8.52±0.31 ^a	8.42±0.49 ^a	8.50±0.40 ^a	8.53±0.54 ^a
LYM (%)	72.00±5.00 ^a	69.92±69.92 ^{ab}	64.12±7.01 ^{ab}	57.68±10.36 ^b
LYM# ($\times 10^3/\mu\text{L}$)	13.55±2.76 ^a	13.18±4.15 ^a	9.27±2.56 ^a	10.30±3.55 ^a
NEUT (%)	28.00±5.00 ^a	30.08±6.92 ^{ab}	35.88±7.01 ^{ab}	42.32±10.36 ^b
NEUT# ($\times 10^3/\mu\text{L}$)	5.33±1.92 ^a	5.77±2.77 ^a	5.10±1.04 ^a	7.28±1.62 ^a
SGOT (U/L)	227.78±33.47 ^c	111.02±9.83 ^a	113.56±18.38 ^a	178.96±46.48 ^b
SGPT (U/L)	54.66±8.22 ^a	46.25±8.26 ^a	43.33±8.46 ^a	53.58±18.24 ^a

Values are presented as mean±SD. Values with different superscripts in the same column are statistically significantly different ($p < 0.05$).

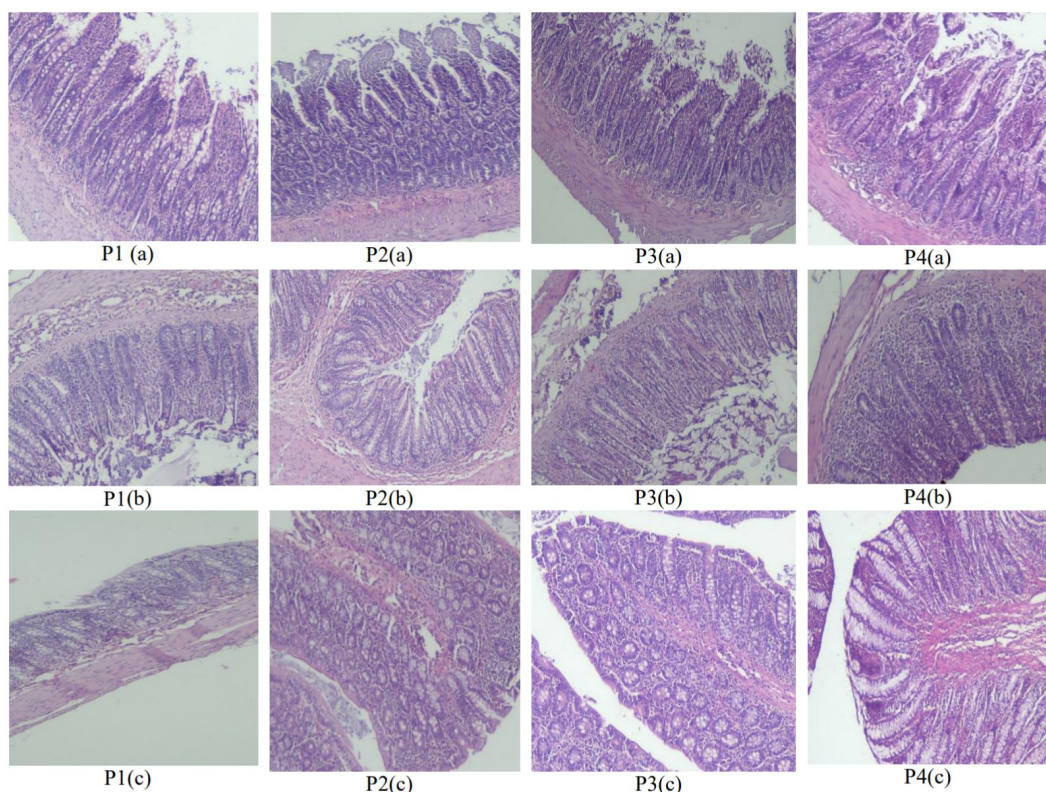


Figure 1. Microscopy of ileum (a), caecum (b), colon (c).

Table 3. Quantitative data of intestinal morphology of rats

Intestinal tract	Parameter (μm)	Group			
		P1	P2	P3	P4
Ileum	Epithelial height	16.97 \pm 4.06 ^a	23.17 \pm 19.49 ^a	15.69 \pm 6.52 ^a	20.72 \pm 2.77 ^a
	Villous height	444.46 \pm 141.11 ^a	518.96 \pm 162.78 ^a	373.04 \pm 65.01 ^a	379.14 \pm 68.43 ^a
Caecum	Epithelial height	14.18 \pm 6.12 ^a	17.44 \pm 1.33 ^a	16.03 \pm 2.06 ^a	18.54 \pm 7.22 ^a
	Villous height	350.48 \pm 25.36 ^a	397.26 \pm 93.82 ^a	333.14 \pm 82.93 ^a	414.72 \pm 87.65 ^a
Colon	Epithelial height	18.27 \pm 4.65 ^a	17.47 \pm 1.89 ^a	17.69 \pm 1.51 ^a	22.21 \pm 5.81 ^a
	Villous height	397.44 \pm 75.38 ^a	388.35 \pm 19.41 ^a	286.01 \pm 63.22 ^a	372.64 \pm 96.82 ^a

Values are presented as mean \pm SD. Values with different superscripts in the same column are statistically significantly different ($p < 0.05$).

3.3 Populations of lactic acid bacteria, *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* in the digesta, feces of rats and compatibility of multi-strain probiotic

The populations of lactic acid bacteria (LAB) in rats' digesta and feces after consuming multi-strain probiotics increased to 10^5 , 10^6 , and 10^7 CFU (Table 4). *Lactiplantibacillus plantarum* and *L. paracasei* were observed in the probiotic group (P3 and P4) with an average growth of 10^5 , 10^6 , and 10^7 CFU/mL. These results showed that *L. plantarum* and *L. paracasei* in the formulation of single-strain and multi-strain survived in the rats' digestive systems. The result was supported by a study done by Rahayu et al. (2019) which showed that *L. plantarum* subsp. *plantarum* (FNCC-0250 and Dad-13) survived in a gastric environment. This study showed that P1 and P2 already have commensal lactic acid bacteria in the gastrointestinal tracts of rats, although these groups did not consume any probiotics. Multi-strain probiotic shows compatibility if the population of multi-strain that survive in the colon or after excretion through feces does not decrease much once ingested in the body. In this research, the average population of *L. plantarum* and *L. paracasei* in the multi-strain probiotic group was not significantly different if compared with the single-strain group, which indicates the compatibility between three different strains in the multi-strain group.

3.4 Bacterial isolation from the blood and organs of the rats

Table 5 shows the presence of bacteria in rat's

Table 4. Population of lactic acid bacteria, *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* in the digesta, feces of rats after 28 days treatment.

Group	LAB (log CFU/mL)		<i>L. plantarum</i> (log CFU/mL)		<i>L. paracasei</i> (log CFU/mL)	
	Digesta	Feces	Digesta	Feces	Digesta	Feces
P1	4.91 \pm 0.69 ^a	5.30 \pm 0.13 ^a	nd	nd	5.14 \pm 0.36 ^a	5.04 \pm 0.31 ^a
P2	5.70 \pm 0.11 ^{ab}	5.64 \pm 0.30 ^a	nd	nd	4.93 \pm 0.22 ^a	nd
P3	6.47 \pm 0.80 ^b	5.66 \pm 0.24 ^a	6.57 \pm 0.43 ^a	5.97 \pm 0.18 ^a	6.88 \pm 1.11 ^b	5.77 \pm 0.45 ^b
P4	6.06 \pm 0.65 ^b	6.66 \pm 0.84 ^b	5.95 \pm 0.42 ^a	6.17 \pm 0.40 ^a	7.03 \pm 0.48 ^b	6.56 \pm 0.39 ^b

Values are presented as mean \pm SD. Values with different superscripts in the same column are statistically significantly different ($p < 0.05$). nd: not detected.

organs. The strains detected were presumed to be *L. paracasei*. However, the blood and organs of the control rats contained LAB. This suggested that indigenous bacteria might have been present. According to Gordon et al. (1995), half of normal mice's MLN, liver, and spleen had bacteria or bacteremia.

Table 5. Number of rats containing bacteria in blood and organs.

Sample	LLV media specific for <i>L. paracasei</i>			
	P1	P2	P3	P4
Kidney	2/6*	1/6*	1/6*	2/6*
Blood	4/6*	2/6*	2/6*	4/6*
Spleen	4/6*	3/6*	2/6*	3/6*
Liver	2/6*	1/6*	4/6*	4/6*
Total	41			

a/b: number of positive rats /total number of examined rats.

According to Liong (2008), the translocation of probiotics can affect infections, including bacteremia and septicemia. As a result, verifying the translocation potential of novel probiotics is crucial. A total of 41 bacterial isolates were found in the rats' blood and organs, but only seven isolates were identified as Gram-positive rod cells, which cannot produce catalase and shared similar phenotype characteristics with GMRMP-001.

3.5 Rep-PCR bacteria identification using BOXA1r primer

Seven isolates, GJP2A1, LPP2A3, LPP4A2C, LPP4A2D, DRP4A5A, DRP4A5B, LVP4A1 (Figure 2)

were identified using rep-PCR with BOXA1R primer, it showed that all the isolates have no similarities to genotype characteristics of GMRMP-001. Data analysis by NTSYSpc (Figure 3) showed that the isolates LVP4A1, GJP2A1, LPP2A3, DRP4A5B, DRP4A5A, LPP4A2D have 91.5% similarity with strain *L. paracasei* GMRMP-001 and LPP4A2C have 79% similarity with *L. paracasei* GMRMP-001.

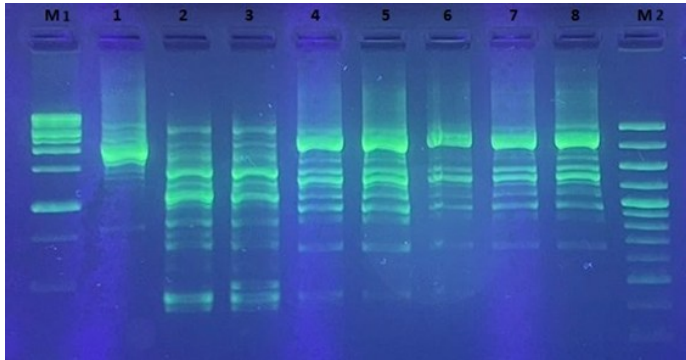


Figure 2. The agarose gel electrophoresis result from rep-PCR using the BOXA1R primer for the 7 bacterial isolates. Lane M1: 1 kb marker, Lane 1: *L. paracasei* GMRMP-001, Lane 2: LPP4A2C, Lane 3: LVP4A1, Lane 4: GJP2A1, Lane 5: LPP2A3, Lane 6: DRP4A5B, Lane 7: DRP4A5A, Lane 8: LPP4A2D, and Lane M2: 100 bp marker.

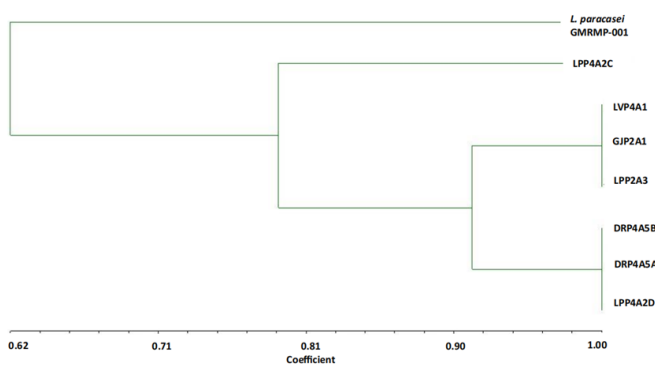


Figure 3. Dendrogram of 8 bacterial isolates from rats after rep-PCR process.

3.6 16S rRNA gene sequencing for the identification of bacteria

The outcomes of the rep-PCR were next processed for genetic analysis by 16S rRNA. Five isolates chosen (*L. paracasei* GMRMP-001, LPP4A2C, LVP4A1, GJP2A1, DRP4A5B) were analyzed by 16S rRNA. This 16S ribosomal RNA (rRNA) gene is recognized as the best method for the taxonomic classification of probiotic strains, as noted by Vankerckhoven *et al.* (2008). A phylogenetic tree was made to confirm the genetic relationships. In Figure 4, GMRMP-001 showed a distant genetic relationship with other isolates (DRP4A5B, LVP4A1, GJP2A1, and LPP4A2C). This suggests that *L. paracasei* GMRMP-001 was not the type of bacteria isolated from the blood and organs. Bacteria found in the samples could be indigenous bacteria from the rat. It can be concluded that bacteria found in the

blood, spleen, kidney, and liver were different from GMRMP-001.

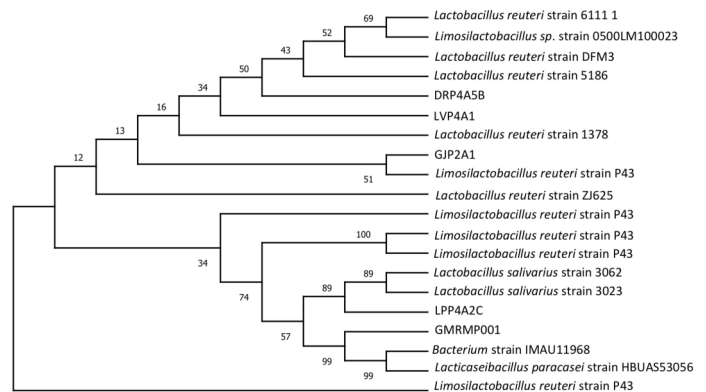


Figure 4. Based on 16S rRNA gene sequencing, the genetic relationship between the isolates from the sample and other bacterial species was analyzed. The numbers in this phylogenetic tree represent the percentage of bootstrap value from 1000 repetitions.

4. Conclusion

The study outcomes indicated that administering a repeated dose of the P4 group did not lead to any toxic effects in rats, even at a high concentration. It can be concluded that *L. paracasei* GMRMP-001 at high doses did not cause bacterial translocation into the blood and other organs of the rats. The average populations of *L. plantarum* and *L. paracasei* in the multi-strain probiotic group showed compatibility with multi-strain probiotics.

Conflict of interest

The authors declare no conflict of interest.

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