

Original Research Paper

Safety Assessment of Indigenous Probiotic Strain *Lactobacillus plantarum* Mut-7 Using Sprague Dawley Rats as a Model

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Abstract: *Lactobacillus plantarum* Mut-7 is a probiotic candidate isolated from *gatot*, traditional fermented cassava from Java, Indonesia. This study aimed to evaluate safety aspects of high dose consumption of *L. plantarum* Mut-7 (10^{11} CFU/ml/day) on Sprague Dawley rats for 21 days. Twenty four female rats were randomly divided into 4 groups; initial condition group (P.0), control group (P.1), skim milk group (P.2) and probiotics group (P.3). All groups followed adaptation phase of 7 days, followed by treatment phase of 21 days for P.1, P.2 and P.3. The results showed that supplementation of high dose of *L. plantarum* Mut-7 did not have detrimental effects on general health, organ weight, hematology and histology parameters of treated rats. Feed intake and body weight showed no significant difference between groups. *L. plantarum* Mut-7 can survive in gastrointestinal tract of rats, resulting in an increased population of *L. plantarum* in the fecal matter and the digesta of treated rats. Bacterial translocation of *L. plantarum* Mut-7 was not detected in the blood and organ of treated rats as confirmed by rep-PCR with BOXAIR primer and further 16S RNA gene sequencing analysis. Twenty-six isolates from blood and organs of treated rats had low-level similarity (<75%) to that of *L. plantarum* Mut-7, with 10 isolates were further analyzed and found that none of them belong to *L. plantarum*. Although this study was limited to the use of animal study, the findings are useful to support the safety assessment of the use of *L. plantarum* Mut-7 as a probiotic according to the abovementioned parameters.

Keywords: Safety Assessment, Probiotic, Bacterial Translocation, *L. plantarum* Mut-7

Introduction

Probiotics are live organisms that, when consumed in sufficient amounts, confer beneficial effects on the host (FAO/WHO, 2002). Most probiotics belong to Lactic Acid Bacteria group (LAB) such as *Lactobacillus* spp. and some other groups such as *Bifidobacterium* group, *Saccharomyces*, *Streptococcus* and *Lactococcus*. However, some species of *Lactobacillus* and *Bifidobacterium* are not natural microflora and can adapt to conditions in the gastrointestinal tract to withstand the acidic conditions

of the stomach/digestive tract bile salts and to survive pathogens (Emmawati, 2014; Rahayu *et al.*, 2015).

In recent years, Lactic Acid Bacteria (LAB) has received increasing attention due to their benefits for humans. LAB has long been used for traditional food fermentation (Zhou *et al.*, 2000). A study by Rahayu (2003) reported that 109 of 194 new LAB strains isolated from 21 different traditional Indonesian fermented food were classified as *Lactobacillus*, particularly dominated by *Lactobacillus plantarum-pentosus*. *L. plantarum* Mut-7 is a potential local probiotic strain from *gatot*, a fermented cassava product

that is widely consumed in Java, Indonesia. Subsequently, Rahayu *et al.* (2019a) also mentioned that Indonesian adults tend to have a very high prevalence of 95% of *L. plantarum* which could be linked to consumption of fermented foods as part of Indonesian diet. Therefore, *L. plantarum* can be considered as a potential probiotic for Indonesian population.

Multiple studies have tested favorable characteristics of *L. plantarum* Mut-7 for probiotic functionality such as it may provide health effects including lowering cholesterol body improve immune system through an increase in phagocytic activity of macrophages, is able to survive in a pH 2, has the ability to inhibit the growth of pathogenic bacteria such as *Escherichia coli*, *Clostridia* and *Salmonella* in the feces (Ngatirah *et al.*, 2000; Lestari *et al.*, 2013).

Lactobacillus spp. used as probiotics are generally classified as GRAS (Generally Recognized as Safe) (Gharaei and Eslamifar, 2011). However, probiotic effects are strain-specific which consequently needs a strain by strain basis for its safety assessment (Kemgag *et al.*, 2014). Therefore, regardless of the long use of *L. plantarum* in food fermentation without causing adverse health effects, its safety aspects for its potential use as probiotic needs to be studied before further use in commercial scale.

Several significant risks that could arise when probiotics are supplemented in humans are potential transmigration, bacteremia, endocarditis potential, gastrointestinal toxicity and antibiotic resistance gene transfer. In recent years, some species of genus *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Enterococcus* and *Bifidobacterium* are often isolated from different types of wound infection. *Lactobacillus rhamnosus*, *L. acidophilus*, *L. plantarum*, *L. casei*, *L. paracasei*, *L. salivarius*, *L. lactis* and *Leuconostoc mesenteroides* are some examples of Lactobacilli isolated from bacterial endocarditis. Additionally, *Pediococcus acidilactici*, *Bifidobacterium eriksonii* and *Bifidobacterium adolescentis* have been isolated from bloodstream infection and local infection (Ishibashi and Yamazaki, 2001).

Amplification of bacterial DNA with repetitive PCR (rep-PCR) has been recognized as one of a simple PCR technique with a discriminatory power, low cost, very suitable for the identification of bacterial strains and reliable method for classifying and typing gram-negative and gram-positive bacteria (Gevers, 2001). Berthier and Ehrlich (1998) stated that the intergenic spacer region (RISA/ITS-PCR) can be used to discriminate between species of *Lactobacillus* with close genetic relationship, namely *L. paraplantarum*, *L. pentosus* and *L. plantarum* using a primer with a target gene in 16S/23S rRNA spacer region. The principle of RISA-PCR technique is through amplification in the intergenic small (16S) and sub-units of large rRNA gene (23S) in the rRNA operon as a target region oligonucleotide primer (Yu and Mohn, 2001). Furthermore, 16S rRNA is a gene that is

generally targeted for DNA sequencing analysis. This technique has a high discriminatory power of 100% type ability and good reproducibility. A similarity score of <97% is interpreted that an isolate represents a new species, while >97% similarity score can be either a new species or an alternative to a new cluster in the previous taxonomy (Janda and Abbott, 2007). Additionally, if an isolate has a minimum 99% similarity, it is identified as a similar strain or species to that of reference species of the gene bank.

The aim of this study, was to evaluate the safety of *L. plantarum* Mut-7, using Sprague Dawley rats as a model, by orally introducing high doses (10^{11} CFU) of *L. plantarum* Mut-7 for 21 days. Some parameters were analyzed including body weight change, organ weight, hematological and histological parameters, such as SGOT, SGPT, morphology of gastrointestinal tract and bacterial analysis in the organs (blood, lungs, heart, spleen, liver and kidneys). The possibility of translocation of *L. plantarum* Mut-7 to the blood and organs of the rats was also investigated using Rep-PCR methods with BOXAIR primer and further 16S RNA gene sequencing analysis.

Materials and Methods

Bacterial Strain and Cell Production

L. plantarum Mut-7 was obtained from the Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. Biomass production was carried out by inoculation of *L. plantarum* Mut-7 into Peptone Glucose Yeast extract broth and incubation for 48 h. The culture was then centrifuged and the pellet was resuspended in 10% skim milk solution at concentration of 10^{11} CFU/ml.

Experimental Design

This research adhered to the Declaration of Helsinki guideline and had been approved by the Medical and Health Research Ethics Committee (MHREC) according to ethical clearance number 331/KEC-LPPT/X/2015. Twenty four female Sprague Dawley rats (albino Norway/*Rattus norvegicus*) aged 8 weeks with body weight 200 ± 20 g were housed individually in stainless steel cage. A 12 h light-dark cycle and a temperature 25°C were maintained in this study. Animals were fed with 15 g of AIN-93 M without tert-butylhydroquinone diet and water ad libitum (Reeves *et al.*, 1993 with modification).

The rats were adapted for one week in experimental condition and subsequently assigned into four groups. First group, named as initial condition group (P.0) followed only adaptation phase of 7 days and were humanely euthanized on day 8. The other three groups

were control group (P.1), skim milk group (P.2) and probiotics group (P.3) followed adaptation phase of 7 days and 21 days of treatment fed with standard based diet. Group P.1 did not receive any other treatment than standard feeding, while P.2 and P.3 were supplemented with skimmed milk 1ml 10% and 1ml cell suspension of *L. plantarum* Mut-7 10^{11} CFU/rats/day in skimmed milk 10%, respectively. On day 29, the rats were euthanized

Anatomy of organ from each rat was checked and recorded. Organ weight index was expressed as actual organ weight (mg) divided by body weight (g). Feed intake and body weight were measured weekly. Furthermore, activity and behavior of each rat were observed and recorded daily. Their blood and tissue (kidney, liver, lung, spleen and heart) samples were collected aseptically for further analysis.

Biochemical, Hematological and Gut Histological Analyses

Blood samples were analyzed for Serum Glutamic Oxaloacetic Transaminase (SGOT) and Serum Glutamic Pyruvic Transaminase (SGPT). Blood serum was obtained by centrifugation and reacted with enzyme reagents and reagent starter. Absorbance was measured using MicroLab 300 by spectrophotometric method at wavelength 340 nm (Steppe *et al.*, 2014).

Blood samples were obtained by orbital sinus on the eyes and collected into an EDTA treated tube for further hematological analysis, including White Blood Cells (WBC) (leukocytes, lymphocytes and neutrophils), number of Red Blood Cells (RBC), Platelet Count (PLT), Hematocrit (HCT), Hemoglobin (HGB), Mean Corpuscular Volume average (MCV), number of mean corpuscular hemoglobin (MCH) and concentration of Mean Corpuscular Hemoglobin (MCHC). Hematological tests aimed to detect abnormal function of red blood cells (anemia and leukemia), to detect systemic disorders (liver and kidney) and the presence of infection. Hematology analyzer is based on the principle of flow cytometer by measuring the number and properties of the cells coated by the flow of liquid through the narrow opening. Thousands of cells flowed through the gap allowing the cells to pass one by one, then the number of cells and the size is calculated (Zhou *et al.*, 2000).

Ileum, caecum and colon morphologies were analyzed qualitatively using a microscope and by measuring epithelial height, villous height and mucosa thickness. The tissue samples were stained using Hematoxylin Eosin (HE) (Shu *et al.*, 2000).

Microbial Analyses of Fecal Matter, Digesta, Blood and Organs in Rats

Analysis of LAB and *L. plantarum* population in the digesta and feces referred to the method by Frias *et al.*

(2009) with modification, Yakabe *et al.* (2009), Zhou *et al.* (2000) and Rahayu *et al.* (2019b).

Fecal matter and digesta samples (1 g) were homogenized and diluted by serial dilution with 9 mL of phosphate saline buffer (PBS). For each dilution, 0.1 mL of sample was inoculated in MRS agar (Oxoid, UK), 3 g CaCO₃, 1 mL NaN₂, 1 L distilled water and *Lactobacillus plantarum* Selective Medium (LPSM), 15 g bacteriological agar (Oxoid, UK), 20 g sorbitol, 10 g bacteriological peptone (Oxoid, UK), 10 g beef extract (Oxoid, UK), 5 g yeast extract (Oxoid, UK), 5 g CH₃COONa, 2 g K₃PO₄, 0,1 g MgSO₄, 0,05 g MnSO₄, 0,02 g Bromocresol purple and 2 mL antibiotic (Ciprofloxacin IV 0,2%).

To evaluate possible bacterial translocation in blood and organs of the rats, 0.1 mL sample from the liver, kidneys, lungs, heart, spleen and blood of each rat was enriched into MRS broth medium for 1 h and 24 h. Growth with streak plate method on MRS agar and LPSM were incubated at 37°C for 48 h. Colonies appearing on MRS were suspected as LAB, while those appeared on LPSM were suspected as *L. plantarum* (Zhou *et al.*, 2000 with modification; Rahayu *et al.*, 2019b).

Isolation of Bacterial Genome DNA

Isolation of bacterial DNA from blood and organs of rats and nine strains of *L. plantarum* was performed using DNA mini kit from GeneaidPrestoTM. The isolated DNA is stored in a 1.5 mL microtube at -20°C.

Amplification of Repetitive Polymerase Chain Reaction (Rep-PCR)

PCR-mix Ready to Go (RTG) was used as materials for the PCR amplification reaction. Each ampoule of PCR-mix RTG reaction was added with 1 µL DNA, primer 1 µL BOXA1R (5'CTACGGCAAGGCAAGGCGACGCTGACGCTGACG-3') and 23 µL Nuclease free water. This PCR amplification consisted of several stages: (1) initial denaturation (94°C, 4 min) 1 cycle, (2) 30 cycles of denaturation (92°C, 1 min), annealing (50°C, 1 min) and extension (68°C; 8 min), (3) 1 final cycle of 65°C, 10 min.

Amplification of Ribosomal Intergenic Spacer Analysis (RISA)

Each ampoule of PCR-mix RTG reaction was added with 1 µL DNA, primer 1 µL S926f (59-CTYAAAKGAATTGACGG-39), 1 µL L189r (59-TACTGAGATGYTTMARTTC-39) and 22 µL Nuclease free water. PCR amplification using a DNA thermal cycler consisted of several steps: (1) initial denaturation (95°C, 1 min) 1 cycle, (2) 35 cycles of denaturation (94°C, 1 min), annealing (50°C; 1.5 min) and extension (72°C; 2 min), (3) one end of cycle 72°C; 5 min.

Amplification and Sequence Analysis using 16S rRNA Gene

Each ampoule of PCR-mix RTG reaction was added with 1 µL DNA, 1 µL primer 27F (AGAGTTTGATCCTGGCTCAG), 1 µL primer 1492R (GGTACTTGTTACGACTT) and 22 µL Nuclease free water. PCR amplification using a DNA thermal cycler consisted of several steps: (1) initial denaturation (96°C, 4 min) 1 cycle, (2) 35 cycles of denaturation (94°C, 1 min), annealing (52°C; 1.5 minutes) and extension (65°C; 8 min), (3) one end of cycle 68°C; 10 min, 4) cooling 12°C; 10 min.

The process of DNA sequencing was done by 1st BASE DNA Sequencing Service, Malaysia. The results of subsequent determination of DNA base sequence were read using DNA baser program. Furthermore, the matching process was done by selecting menu *Alignment* with *Bioedit*. Matching results were used to search for DNA base sequence of genes that are similar or similar as DNA base sequence data for genes from an international gene bank database through the NCBI BLAST method. Based on phylogenetic trees, DNA sequence was used as data reference. DNA sequence data can be retrieved from international databases of NCBI. The selected bacterial DNA sequence was stored as reference isolates using MEGA7 format (version 7.0.14) (Tamura *et al.*, 2011) and followed by neighbor joining method (Saitou and Nei, 1987).

Result and Discussion

Population of LAB in Digesta and Feces

After consumption of *L. plantarum* Mut-7 for 21 days, LAB population in P.3 (probiotic group) showed an increase to max 10⁸ CFU/ml in both feces and digesta. These results are in agreement with those of Rahayu *et al.* (2019b), where the numbers of LAB in digesta and feces were 10⁷ CFU/ml and 10⁸ CFU/ml after administration of *L. plantarum* Dad-13. These findings indicated that *L. plantarum* survive gastrointestinal tract into feces. Ability to survive in gastrointestinal tract is an important characteristic of probiotic to be able to give beneficial effects to the host as defined by FAO/WHO (2002).

Survival rates of *L. plantarum* Mut-7 in gastrointestinal tract can be seen in Table 1. *L. plantarum* was only detected in probiotic group (P.3) with an average growth of 10⁴-10⁵ CFU/ml and promotes the growth of LAB in the digestive tract of probiotic group. These results are consistent with studies by Rahayu *et al.* (2015) where, in *in vitro* testing of *L. plantarum* Mut-7, it was able to survive in pH 2 gastric acid, a 3% concentration of bile salts and to inhibit pathogenic bacteria.

General Health Indicator and Biochemical and Hematological Parameters

All rats in group P.1, P.2 and P.3 survived until day 28 of the experiment. Physiological conditions were consistent and showed no adverse effects during the consumption period of probiotics. This consistency in physiological conditions was used as an indicator of infection caused by microbial metabolite production and release of cytokines which may affect central nervous system (Kanra *et al.*, 2006).

The results of body weight and feed intake showed no abnormal changes were recorded. The organ weight index ratio can be seen in Table 2. The mean index of organ weights between different groups did not experience a significant difference (P>0.05) and thus did not result a significant increase in organ weight ratio. It shows that the administration of probiotics for 28 days did not lead to a bacterial infection in rats' organs

The results of hematological tests showed no significant increase (P>0,05) in any of the levels of hematologic parameters after ingestion of *L. plantarum* Mut-7 as shown in Table 3. SGOT and SGPT were two parameters to assess hematological condition of rats. SGOT and SGPT were higher than normal values in all groups, showing that elevated levels of SGOT and SGPT had allegedly caused hepatocyte damage which had occurred since initial conditions. Oxidative stress can also lead to high levels of SGOT and SGPT due to an accumulation of metabolites in the body disturbing the balance of free radicals and antioxidants. It can be expected that the increase in SGOT and SGPT levels above the normal values was not caused by the consumption of *L. plantarum* Mut-7 at a high dose.

Table 1: The population of LAB and *L. plantarum* at the end of the treatment

| Group | LAB count (Log CFU/ml) | | | | | | <i>L. plantarum</i> count (Log CFU/ml) | | | | | |
|-------|------------------------|------|------|-------|------|------|--|------|------|-------|------|------|
| | Digesta | | | Feces | | | Digesta | | | Feces | | |
| | Mean | Min | Max | Mean | Min | Max | Mean | Min | Max | Mean | Min | Max |
| P.0 | 5,32 | 4,08 | 6,79 | 5,77 | 3,78 | 6,90 | nd | nd | nd | nd | nd | nd |
| P.1 | 4,64 | 3,00 | 6,16 | 5,29 | 4,32 | 6,00 | nd | nd | nd | nd | nd | nd |
| P.2 | 5,77 | 4,46 | 6,38 | 6,37 | 5,33 | 7,48 | nd | nd | nd | nd | nd | nd |
| P.3 | 7,18 | 6,25 | 8,16 | 8,16 | 7,29 | 8,48 | 4,85 | 3,65 | 5,58 | 4,90 | 3,81 | 6,48 |

Note: nd = not detected

Table 2: Organ weight index per group

| Group | Heart | Spleen | Lungs | Liver | Kidney |
|-------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| P.0 | 0,55 ^a ±0,06 | 0,68 ^a ±0,13 | 0,89 ^a ±0,37 | 3,07 ^a ±0,37 | 0,75 ^a ±0,16 |
| P.1 | 0,55 ^a ±0,06 | 0,59 ^a ±0,07 | 0,90 ^a ±0,35 | 2,87 ^a ±0,38 | 0,80 ^a ±0,10 |
| P.2 | 0,52 ^a ±0,04 | 0,47 ^a ±0,05 | 0,76 ^a ±0,10 | 2,53 ^a ±0,02 | 0,80 ^a ±0,02 |
| P.3 | 0,56 ^a ±0,03 | 0,50 ^a ±0,11 | 0,77 ^a ±0,14 | 2,52 ^a ±0,04 | 0,79 ^a ±0,10 |

Note: Different superscript notation in the same column indicate significant difference (P>0.05) + SEM = standard error

Table 3: Results of blood hematology tests (mean ± SD)

| Parameter | P.0 | P.1 | P.2 | P.3 | Ref |
|------------------------------|-------------------------|-------------------------|-------------------------|---------------------------|-------|
| WBC (×10 ³ /μL) | 7,7 ^a ±1,08 | 7,5 ^a ±0,85 | 8,1 ^a ±0,53 | 7,2 ^a ±0,95 | 5,3 |
| RBC (×10 ⁶ /μL) | 6,8 ^a ±0,31 | 7,2 ^a ±0,37 | 7,9 ^a ±0,46 | 7,3 ^a ±0,29 | 8,2 |
| HGB (g/dL) | 13,5 ^a ±0,46 | 14,0 ^a ±0,58 | 14,7 ^a ±0,48 | 13,9 ^a ±0,34 | 14,6 |
| HCT (%) | 41,1 ^a ±1,35 | 41,9 ^a ±1,87 | 44,2 ^a ±1,86 | 42,3 ^a ±1,08 | 44,6 |
| MCV (fL) | 60,3 ^a ±1,24 | 57,9 ^a ±0,83 | 56,3 ^a ±1,26 | 58,4 ^a ±0,86 | 54,4 |
| MCH (pg) | 19,8 ^a ±0,53 | 19,4 ^a ±0,47 | 18,7 ^a ±0,60 | 19,2 ^a ±0,51 | 17,78 |
| MCHC (g/dL) | 32,8 ^a ±0,55 | 33,5 ^a ±0,53 | 33,2 ^a ±0,37 | 32,9 ^a ±0,51 | 32,72 |
| PLT (×10 ⁴ /μL) | 7,3 ^a ±12,82 | 60,4 ^a ±4,78 | 77,7 ^a ±9,86 | 68,4 ^a ±6,47 | 87,0 |
| LYM (%) | 65,7 ^a ±3,96 | 55,5 ^a ±6,06 | 70,5 ^a ±3,99 | 67,6 ^a ±4,67 | 76,8 |
| LYM# (×10 ² /μL) | 50,6 ^a ±6,32 | 40,7 ^a ±5,98 | 57,5 ^a ±5,93 | 47,0 ^a ±4,03 | 41,6 |
| NEUT (%) | 34,3 ^a ±3,96 | 44,5 ^a ±6,06 | 29,5 ^a ±3,99 | 32,4 ^a ±4,67 | 24,1 |
| NEUT# (×10 ² /μL) | 27,3 ^a ±6,27 | 34,3 ^a ±6,08 | 23,1 ^a ±2,89 | 24,8 ^a ±6,76 | 10,3 |
| SGOT (×10U/l) | 12,7 ^a ±9,06 | 9,8 ^a ±6,85 | 17,1 ^b ±1,66 | 14,96 ^{ab} ±1,16 | 8,9 |
| SGPT (×10U/l) | 7,45 ^a ±8,61 | 7,12 ^a ±6,37 | 8,65 ^a ±6,49 | 7,64 ^a ±6,77 | 6,8 |

Note: Different superscript notation in the same column indicate significant difference (P>0.05) + SEM = standard error. Ref (reference (Steppe *et al.*, 2014)), WBC (leukocytes), LYM (lymphocytes), NEUT (neutrophil), RBC (red blood cells), PLT (platelet count), hematocrit (HCT), hemoglobin (HGB), the mean corpuscular volume average (MCV), the number of mean corpuscular hemoglobin (MCH) and the concentration of mean corpuscular hemoglobin (MCHC)

Gut Histology Analysis

The results showed that after consumption of *L. plantarum* Mut-7, there was no inflammation which subsequently triggered further infection. An inflammation is characterized by changes in the structure of ileum such as reddish spots, enlarged globules of mucosa, thinning of the mucosa, lengthened intestinal villi, thickening of bowel wall and widening of space between villi and crypts (Shackelford and Elwell, 1999).

Figure 1 shows epithelial cells in healthy status. The mechanism of bacterial attachment (adhesion) first took place in the mucous layer as a bond between the structure of the bacteria and specific receptors on the surface of the epithelial cells in which glycoconjugates attach to the side of the oligosaccharide chains of membrane microvilli (Ouwehand and Salminen, 2003). The ability of a bacteria strain to attach to mucosal layer may vary, depends on the strain type. Pathogenic bacteria causing erosion and damage to the mucosal lining of the epithelial cell surface.

Bacterial Analyses of Blood and Organs of Rats

The results of microbiological analysis (Table 4) using MRS with the addition of CaCO₃ detected bacterial colonies in the blood of both P.0 (initial condition group) and P.3 (probiotic group). Moreover, bacterial colonies were also detected in the heart, spleen, liver, lungs and kidneys in all groups. Bacterial colonies

detected in MRS media were suspected as LAB. The highest bacterial translocation in organs occurred in liver with 79% occurrence in all rats. Rahayu *et al.* (2019b) conducted an evaluation for probiotic candidate, *L. plantarum* Dad-13, in which bacterial colony detected in MRS media were suspected as LAB. According Frizzo *et al.* (2010), an organ incubated on an agar medium is not free from microbes which renders a possibility for other bacteria to grow. A positive culture in organs and blood of rats treated with probiotic did not indicate that the probiotics are responsible for the translocation because translocation of LAB can also be found even in untreated animals (Trevisi *et al.*, 2007).

Nine strains of *L. plantarum* from FNCC (Food and Nutrition Culture Collection UGM, Indonesia) were evaluated by RISA-PCR and did not produce a specific fingerprint patterns between the strains (Fig. 2A.) Limited number of bands and separation of the bands did not clearly show the differences between strains of bacteria. Limited number of bands obtained from RISA-PCR amplification might be due to the long intragenic spacer region on each strain *L. plantarum* which is relatively similar so it does not form specific fingerprint patterns. Berthier and Ehrlich (1998), states that beside the 16S - 23S spacer, intergenic spacer region of the t-RNA genes can be used to discriminate between species *Lactobacillus* based on the unique fingerprint patterns of t-DNA. However, there is a failure to distinguish very

similar species. RISA-PCR method used in this study proved to be unable to distinguish between strains of *L. plantarum*. In addition, the composition of the target gene amplification (16S-23S ISR) among strains of *L. plantarum* has higher similarity.

The results of the genotyping of nine strains of *L. plantarum* using Rep-PCR method showed satisfactory results (Fig. 2B.). This is shown by the results of PAGE PCR in which fingerprint patterns were different between strains. Differences of bands pattern (fingerprint) at each different strains of *L. plantarum* in the first group of species indicate that Rep-PCR has a high resolution in the analysis of closely related bacterial strains. This

statement is supported by previous studies in distinguishing *L. plantarum* Dad-13 with other strains of *L. plantarum* by finger printing patterns using molecular analysis of rep-PCR with primers BOXA1R (Rahayu *et al.*, 2019b). According Brusetti *et al.* (2008), BOX-PCR has the ability to differentiate strains of the same bacteria even better than ribosomal intergenic spacer analysis (RISA), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and in some cases have a better discriminatory power of pulsed field gel electrophoresis (PFGE), although generally less good in terms of reproducibility.

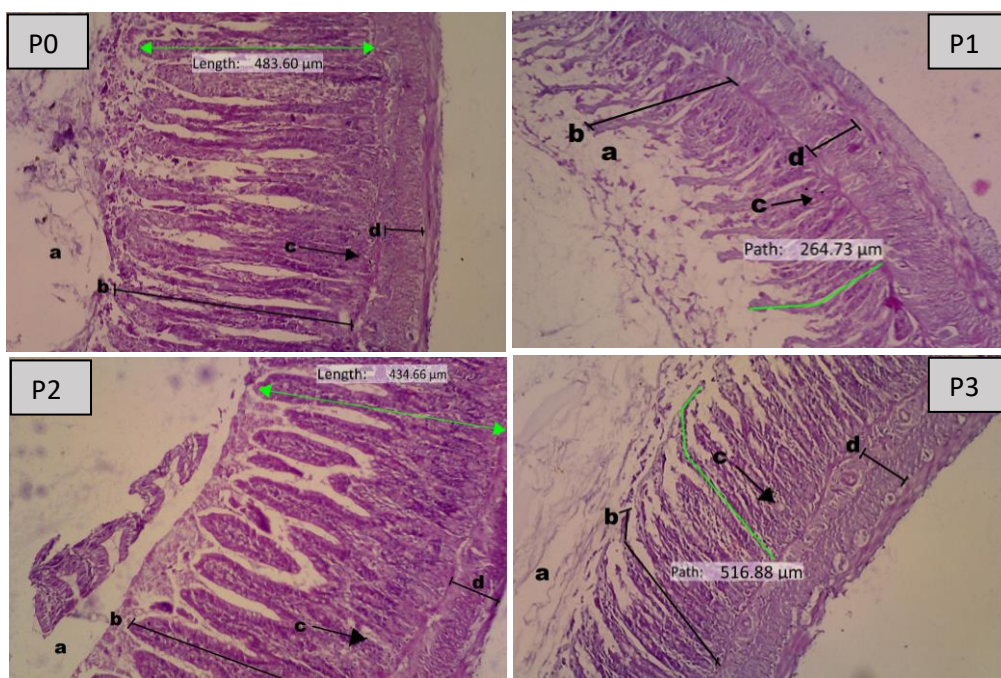


Fig. 1: Microscopy H&E ileum; **Note:** Ileum: a. lumen, b. mucosa, c. muscularis mucosa, d. sub mucosa

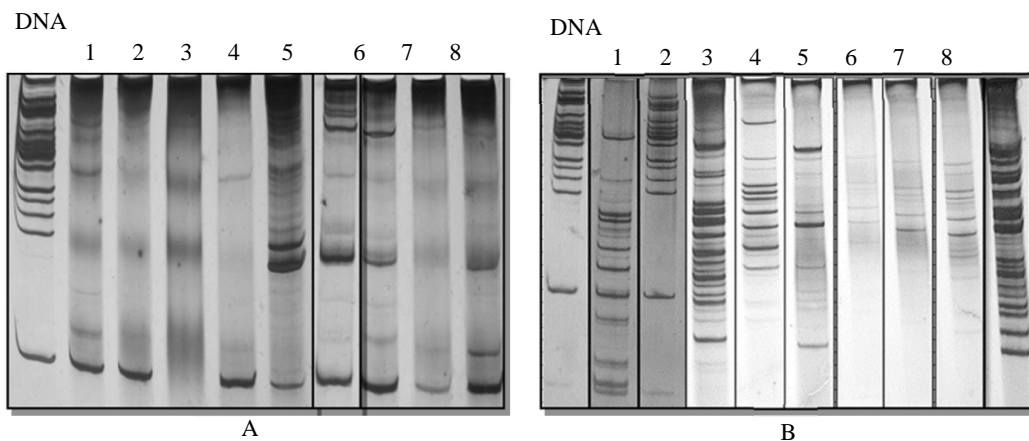


Fig. 2: Result of Polyacrylamide Gel Electrophoresis (PAGE) 8% from PCR Amplification of 9 Strain *L. plantarum* FNCC-UGM

Table 4 shows that in all groups there were no bacterial colonies detected on LPSM. These results indicated that LAB detected in MRS media did not belong to *L. plantarum* colony, particularly *L. plantarum* Mut-7 supplemented to probiotic group. These findings are consistent with previous finding in which *Lactobacillus* do not experience translocation by itself, but can occur when intestinal mucosa is interrupted or when the immune system is unable to control translocation of pathogenic microorganisms into the bloodstream, causing sepsis or local infection (Kemgag *et al.*, 2014). Translocation is associated with immune system and damage to the mucosa. When damage occurs to mucosal epithelial cells, IgA secretion occurs in the immune system, which clinically affects probiotic strain (Frias *et al.*, 2009).

Identification of Bacterial Isolated in Blood and Organs of Rats

Total isolates identified in this study were 33 isolates, consisting of 9, 6, 7 and 11 isolates in the following groups P.0, P.1, P.2, P.3, respectively. However, from 33 isolates analyzed by BOX-PCR, only 26 were successfully amplified by 8% Polyacrylamide Gel Electrophoresis Analysis (PAGE) (Fig. 3). Seven isolates that did not show good results were isolates from group 4.0 (4 isolates) and P1 (3 isolates), due to the reproducibility ability of BOX-PCR method. According to Ranjbar *et al.* (2014), the reproducibility of Rep-PCR can be influenced by the PCR reagents, thermal cycle

and gel electrophoresis conditions. Furthermore, PAGE and silver staining can provide better resolution fragment band than agarose gel but may cause low reproducibility due to gel staining procedure (Bruseti *et al.*, 2008). The number of bands from BOX-PCR analysis of 26 bacterial isolates were 353 bands with an average of 13 bands/isolates. The observation of the pattern of bands (fingerprint) of each isolate was compared to those of *L. plantarum* Mut-7. None of the isolates in each group showed similarities to *L. plantarum* Mut-7. The pattern of bands produced by amplification with BOX-PCR produce are quite complex to discriminate on the level of subspecies, while the presence or absence of one or more bands in one cluster generates heterogeneity among strains clearer than by using another primer (Gevers, 2001). BOX-PCR is not only suitable for identification purposes but also for isolates classification, although only isolates with high similarity can be shown. As for the band patterns that are not identical to BOX-PCR molecular typing techniques require further analysis, such as 5 isolates of P.0 (I1BD, I1LR, I3LR, I4LR) and P.1 (II1LR, II3LR, II4LR).

Based on fingerprint patterns by BOX-PCR analysis, 26 isolates of *L. plantarum* and strain Mut-7 having a similarity level between 54%-90% (Fig. 4). Rep-PCR is used as a genotyping method when the similarity level reaches >95% or 93% (Nucera *et al.*, 2013; Gevers, 2001). Therefore, it can be considered that among 26 isolates there was no resemblance of identical genes to bacterial strain *L. plantarum* Mut-7.

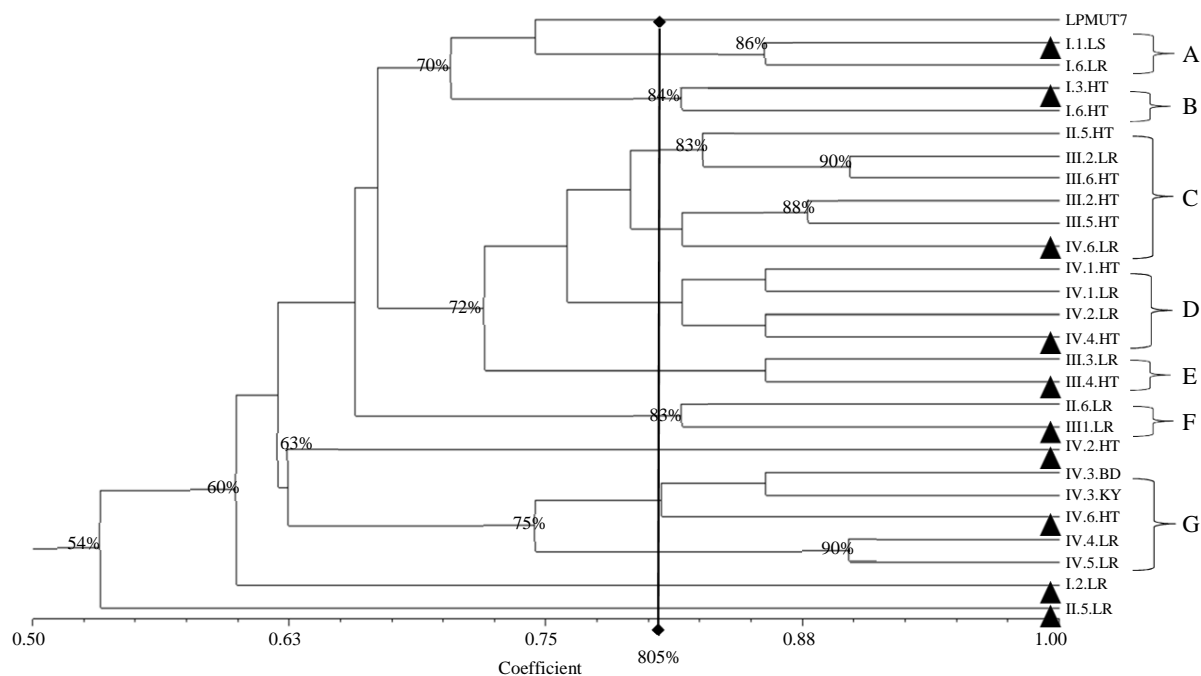


Fig. 3: Result of Polyacrylamide Gel Electrophoresis (PAGE) 8% from 26 Isolate Bacteria with BOX-PCR

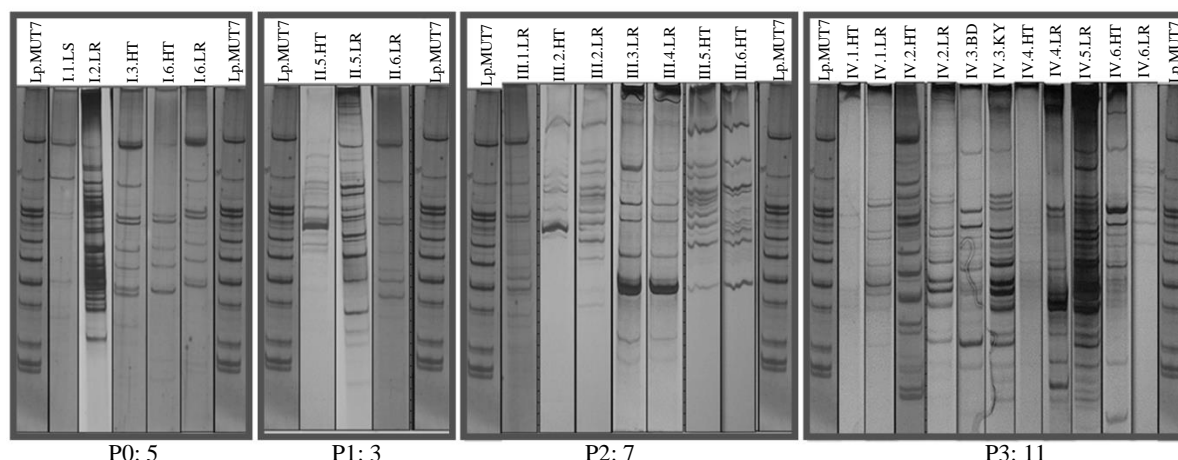


Fig. 4: Dendrogram of 26 Isolates from Organ and Blood Rats Using Rep-PCR with Primer BOX A1R (LS: Lungs; LR: Liver; HT: Heart; BD: Blood; KY: Kidney; I: Group 1; II: Group 2; III: Group 3; IV: Group 4; 1-6: Number of Rat)

Table 4: Incidence of bacterial colonies detected on blood and organs

| | MRS media (LAB) | | | | LPSM (<i>L. plantarum</i>) | | | |
|---------------|-----------------|-----|-----|-----|------------------------------|-----|-----|-----|
| | P.0 | P.1 | P.2 | P.3 | P.0 | P.1 | P.2 | P.3 |
| Blood | 2/6 | 0/6 | 0/6 | 1/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| Heart | 2/6 | 1/6 | 3/6 | 4/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| Liver | 4/6 | 5/6 | 4/6 | 5/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| Lungs | 4/6 | 5/6 | 2/6 | 4/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| Kidney | 3/6 | 2/6 | 2/6 | 3/6 | 0/6 | 0/6 | 0/6 | 0/6 |
| Spleen | 3/6 | 1/6 | 5/6 | 1/6 | 0/6 | 0/6 | 0/6 | 0/6 |

Note: a/b = the positive animals/ total examined animals

Identification of Bacteria based on 16S rRNA Gene Sequencing

Results of analysis of the 16S rRNA gene of the 10 isolates were uploaded into the database Genebank (www.ncbi.nlm.nih.gov/BLAST) and analyzed for sequence homology with species of reference. Based on identification of isolates with 16S rRNA gene analysis, 8 of 10 isolates belong to genus *Lactobacillus* (similarity level 87%-96%), while 2 were *Enterococcus*. Four isolates were classified as *Lactobacillus murinus* (similarity >97.5%) and identified as a strain. Other isolates (16) which were not sequenced were analyzed by amplification with BOX-PCR (Fig. 4).

The mechanism of bacterial translocation from genus *Lactobacillus* could not be determined because the histopathological analysis of intestine did not indicate any presence of inflammation, while interference of microvilli intestine was not analyzed. Therefore, possibility of microbial growth in the gut could not be certainly explained although the number of bacteria in the guts of rats treated with *L. plantarum* Mut-7 (log 8.16 CFU/g) increased compared to those of control group log 5.88 CFU/g). Previous study found that analysis of whole-genome sequence of *L. plantarum*

JDM1 has 126 genes associated with virulence factors (Zhang *et al.*, 2012). Additionally, Ishibashi and Yamazaki (2001) also stated that there were other virulence factors that are owned by a bacteria of genus *Lactobacillus* (*L. rhamnosus*, *L. paracasei* subsp. *paracasei* and other strains), that are glycosidase and protease enzyme activity (arylamide) which can cause damage to the human glycoprotein and the synthesis and fibrin clot lysis, wherein the bacterial strain that has the enzyme can be a cause of infection (endocarditis). Therefore, *L. plantarum* Mut-7 did not have the ability to translocate to organs and blood of rats due to the genes associated with virulence factors do not have the ability to produce these enzymes.

Conclusion

The results of this study showed that supplementation of high doses (10^{11} CFU/ml) of *L. plantarum* Mut-7 for a period of 21 days in Sprague Dawley rats had no deleterious effect on the performance of rat (feed intake, body weight, hematological concentration, physiological and stress markers and gut morphology). *L. plantarum* Mut-7 can survive in gastrointestinal tracts of rats, resulting in a significant increase of the *L. plantarum*

population in the fecal matter and the digest of treated rats. Additionally, consumption of high doses of *L. plantarum* Mut-7 for 21 days did not cause bacterial translocation in the organs or blood of the rats. Although this study was limited to the use of animal study, the findings are useful to support the safety assessment of the use of *L. plantarum* Mut-7 as a probiotic.

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Author's Contributions

Emma Riftyan and Atika Yahdiyani Ikhsani: Carried out the experiments and performed data analysis.

Rosa Amalia Safitri: Contributed in preparing the manuscript.

Yustinus Marsono: Supervised biomolecular analysis.

Tyas Utami and Jaka Widada: Supervised microbiology analysis.

Endang S. Rahayu: Supervised the whole research.

Conflict of Interest

The authors declare no conflict of interest.

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