RESEARCH ARTICLE

Probiotic *Lactobacillus acidophilus* FNCC 0051 Improves Pancreatic Histopathology in Streptozotocin-induced Type-1 Diabetes Mellitus Rats

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Abstract

BACKGROUND: Intestinal microbial dysbiosis and its metabolites can affect the immune activity of intestinal mucosal cells, causing insulitis and pancreatic β-cell death. Probiotic *Lactobacillus acidophilus* plays an important role in reducing inflammatory cytokines, hence improves oxidative stress that affects pancreatic β-cell apoptosis. Current study examined the feature of pancreatic histopathology affected by the administration of probiotic *L. acidophilus* in rats with type-1 diabetes mellitus (DM) induced by streptozotocin (STZ).

METHODS: Twelve rats were induced by STZ at double dose of 50 mg/kgBB before administered with probiotic *L. acidophilus* at a dose of $1.5x10^8$ or $1.5x10^9$ CFU/mL/day, while other 4 rats were used as control. After 21 days of the *L. acidophilus* treatment, the average of fasting blood glucose (FBG) levels of rats were measured, then the pancreatic histopathology was assessed to evaluate the degree of insulitis in islet of Langerhans.

RESULTS: The induction of STZ had been succeeded to increase blood glucose levels, which indicate DM condition. The highest FBG level after 21 days of treatment was found in DM group with glucose level of 512±81.51 mg/dL. The administration of probiotic *L. acidophilus* during 21 days treatment at both dose $1.5x10^8$ and $1.5x10^9$ CFU/mL/day significantly improved pancreatic histopathology (p=0.04 and p=0.034, respectively), with significant decrease on insulitis scores compared to DM group.

CONCLUSION: The administration of *L. acidophilus* at both dose of $1.5x10^8$ and $1.5x10^9$ CFU/mL/day for 21 days can improve pancreatic histopathology of type-1 DM rats induced by STZ, therefore probiotic *L. acidophilus* may be potential as supplementation treatment for type-1 DM.

KEYWORDS: *Lactobacillus acidophilus*, pancreatic histopathology, streptozotocin, type-1 diabetes mellitus

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Introduction

International Diabetes Federation (IDF) have been recorded the number of diabetes mellitus (DM) cases in 2021, that 537 million of adult peoples with DM and estimated to increase to 643 million in 2030.(1) The number of DM cases in Indonesia has more than 10 million consisting of 87-91% of type-2 DM, 7-12% of type-1 DM, and 1-3% are other types of DM.(2) In Indonesia, approximately one million more children and adolescents with type-1 DM and have increased to 0.02819 per 100,000 population from 2000

to 2010.(3) According to these data, the incidence of type-1 DM showed a significant increase, so that important to study about the effectiveness of probiotics administration as supplementation treatment for type-1 DM.

Type-2 DM characterized by insulin resistance as an effect of the impaired insulin production and secretion, while type-1 DM is an autoimmune disease with β-cell destruction.(4,5) Several studies have revealed that gut microbial dysbiosis and the abnormal production of its metabolites associated with the mucosal immune cells disorders cause the pancreatic cell destruction, then contributed to progression and development of type-1 DM.(6) Progression to diabetes is triggered by destruction of pancreatic cells due to cytotoxic T-cell immune responses and infiltration of other immune cells, causing insulitis and ultimately pancreatic cell death.(7) Insulitis occurs in 19% of the total cases of type-1 DM, which identified in the first year after diagnosis, especially in patients aged 0-14 years.(8,9)

Probiotics can improve the intestinal immune system by increasing levels of short-chain fatty acids (SCFA) and crucial to produce glucagon-like peptide-1 (GLP-1) which can improve a condition of DM with pancreatic cells damage.(10,11) In addition, the progress of diabetes is also characterized by improvement of oxidative stress that affects pancreatic cell apoptosis and also the repair of blood sugar parameters. The probiotic strain *Lactobacillus acidophilus* is important in metabolism of gut microbiota, through the reduction of tumor necrosis factor alpha (TNF-α) and interleukin (IL-1β), that are major cytokine of inflammation response.(12,13) Several studies have shown a significant benefit of probiotics in DM. A study showed that the induction of *L. acidopilus* at dose $7.23x10^6$ CFU and *Bifidobacterium lactis* at dose $6.04x10^6$ CFU for 6 weeks in DM rats have improved the oxidative stress thereby preventing apoptosis in pancreatic cells.(14) Streptozotocin (STZ)-induced DM rats treated with *Lactobacillus brevis* at dose of 10^9 CFU for 4 weeks also had pancreatic histopathological features with repair of the islets of Langerhans.(15)

Recent studies have found the effects of probiotic *L. acidophilus* administration on blood glucose levels and lipid profiles, but histopathological features of pancreas organ in type-1 DM rats has not been well evaluated. Thus, in this study was conducted to analyze the effectiveness of probiotic *L. acidophilus* FNCC 0051 to improve the pancreas insulitis condition in type-1 DM *Rattus norvegicus* induced with streptozotocin (STZ), which has a bacteriostatic effect to *L. acidophilus* as an inducer of DM, especially type-1 DM in experimental animals.(16,17)

Methods

Probiotic Bacteria Preparation

Probiotic *L. acidophilus* FNCC 0051 used in this study were obtained from the collection of Center for Food and Nutrition Studies, Universitas Gadjah Mada (Yogyakarta, Indonesia). *L. acidophilus* FNCC 0051 was isolated from human gastrointestinal tract and one of commercial probiotic, which generally used in fermented food products. The bacterial were cultured on 200 mL of de Man-Rogosa Sharpe (MRS) broth media (Merck, Kenilworth, NJ, USATM), and incubated at 37°C for 48 hours under CO_2 enriched condition. The bacterial culture were transferred to a sterile tube containing saline and were measured the optical density using spectrophotometer to adjusted to the McFarland standard, which 0.5 McFarland standard solution is equivalent to $1.5x10^8$ CFU/mL with optical density 0.08-0.1 nm and 5.0 McFarland standard equivalent to $1.5x10^9$ CFU/mL with optical density 0.67-0.7 nm at a wavelength of 625 nm.

Animal Treatment

This was a randomized post-test only control group study, using 16 *Rattus norvegicus* male Sprague Dawley strain. The rats were divided into four groups; Control Group, DM rats (Group 1), DM rats that were given a suspension dose of probiotic *L. acidophilus* at $1.5x10^8$ CFU/mL/day (Group 2), and DM rats that were given a suspension dose of probiotic *L. acidophilus* at $1.5x10^9$ CFU/mL/day (Group 3).

DM rats were induced by STZ (Bioworld, Dublin, Ohio, USATM), a diabetogenic agent that can destroy pancreatic cells, at dose of 50 mg/kgBW intravenously, twice with an interval of 3 days. Two days after the second injection, fasting blood glucose (FBG) level were checked, and rats with FBG levels ≥200 mg/dL were selected. Group 2 and Group 3 were then administered with probiotic *L. acidophilus* at dose of $1.5x10^8$ and $1.5x10^9$ CFU/mL/day, respectively. The protocol of this study was according to the principle outlined in the Declaration of Helsinki 2008 and the 3Rs (replacement, reduction, dan refinement) principal, and had been approved by Research Ethic Committee of Faculty of Medicine and Health Sciences, Universitas Bengkulu (No. 98/UN.30.9/LT/2020).

Body Weight and FBG Examination

Rats body weight were measured before and after acclimatization using a digital scale (Ohaus, Parsippany, NJ, USA). FBG levels were measured on day 5 after STZ

induction and also on day 21 after probiotic treatment. Blood samples were taken from the tail vein of all rats into a microhematocrit tube, then centrifuged and the supernatant were collected to check the FBG levels using an automatic chemistry analyzer (Biobase, Jinan, Shandong, China) at the Clinical Laboratory of Bhayangkara Hospital, Bengkulu.

Histopathological Examination

The histopathology procedures consist of several steps; the fixation using 10% buffer neutral formalin (BNF) solution, followed by the cutting of organ tissue, dehydration, clearing, impregnation/infiltration, embedding, slicing, hematoxylin-eosin (HE) staining, and mounting. For the pancreatic histopathological assesment, the average area islet of Langerhans was counted by randomly selecting 5 visual fields with 100x and 400x magnification by two independence anatomical pathologists, then the area islet of langerhans was confirmed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The insulitis score was determined according to previous study using a scale of 0-4 representing five categories; score 0 referred to no insulitis, score 1 referred to peri-islet insulitis (less than 25% of infiltrated islets), score 2 referred to intermediate insulitis (25% of infiltrated islets), score 3 referred to intra-islet insulitis (between 25% and 75% of infiltrated islets), and score 4 referred to complete islet insulitis (more than 75% of infiltrated islets).(18)

Statistical Analysis

Statistical analysis was performed using the Statistical Program for Social Science (SPSS) for windows version 23 (IBM Corporation, Armonk, NY, USA). The differences of FBG levels between group were analyzed using one way ANOVA test. After the grade of insulitis was obtained, the Shapiro-Wilk normality test was performed to determine the distribution of the data. The results of the normality test showed that the degree of insulitis was not normally distributed, so the Kruskal-Wallis continue with post hoc using Mann-Whitney test were used to analyze the difference in the degree of insulitis and to determine the relationship between groups. The $p<0.05$ was considered as significant.

Results

The rats were acclimatized for 7 days and measured for average of body weight before and after the acclimatization. Based on statistical analysis using the Kruskal-Wallis test, a significant difference in weight loss between groups was found ($p=0.019$). The induction of STZ had been succeeded to increase FBG levels, which indicate DM condition. One way ANOVA test results showed a significant difference in FBG levels between treatment groups, with the highest FBG level after 21 days of probiotic treatment was in Group 1 (group that was administered with multiple dose of 50 mg/kgBW STZ induction) (Table 1).

Histopathological analysis results showed insulitis of the pancreatic islets of Langerhans (Figure 1). In the Control Group, the ovoid shaped cells were seen scattered throughout the pancreatic islets, small mitochondria and the golgi apparatus with many blood vessels, and there was no inflammation in the islets of Langerhans (Figure 1A). In the Group 1 with grade 3 insulitis, 75% of the islets were inflamed with lymphocytes, there were lots of intercellular spaces, the nucleus was smaller and well-defined (Figure 1B). Meanwhile, Group 2 was characterized by a grade of insulitis score of 2 with the appearance of the pancreatic islets of Langerhans being wider than Group 1 and the lymphocytes decreased by 50% (Figure 1C). Furthermore, the Group 3 also had improvement, which was marked by a score of 0 insulitis degree with no lymphocyte cells found in the islets of Langerhans (Figure 1D). The area islet of Langerhans with highest mean area was found in the Control Group (43.92 mm^2) and the lowest mean area was in the Group 1 (17.19 mm^2).

The grade of insulitis in the control group based on histopathological scoring obtained the score of 0, and the mean score of the grade of insulitis in the Group 1, Group 2, and Group 3 were 2.75, 2.00, and 1.25, respectively. Based on the Kruskal-Wallis analysis, the degree of insulitis has a significant difference ($p=0.006$) between the treatment groups (Figure 2). Furthermore, the data were analyzed by Mann-Whitney test to find out which treatment groups had significant differences, and the result showed the significant difference in the degree of pancreatic histopathological insulitis scores between Control Group and Group 1 ($p=0.011$), between Control Group and Group 2 ($p=0.008$),

Table 1. FBG levels of normal rats and type-1 DM rats.

Group	n	FBG Levels (mg/dL)	*p*- value
Control group	4	91.25±9.91	0.000*
Group 1	4	512±81.51	
Group 2	4	427.25±40.70	
Group 3	4	430±29.41	

*$p<0.05$ is considered as significant, tested with one way ANOVA.

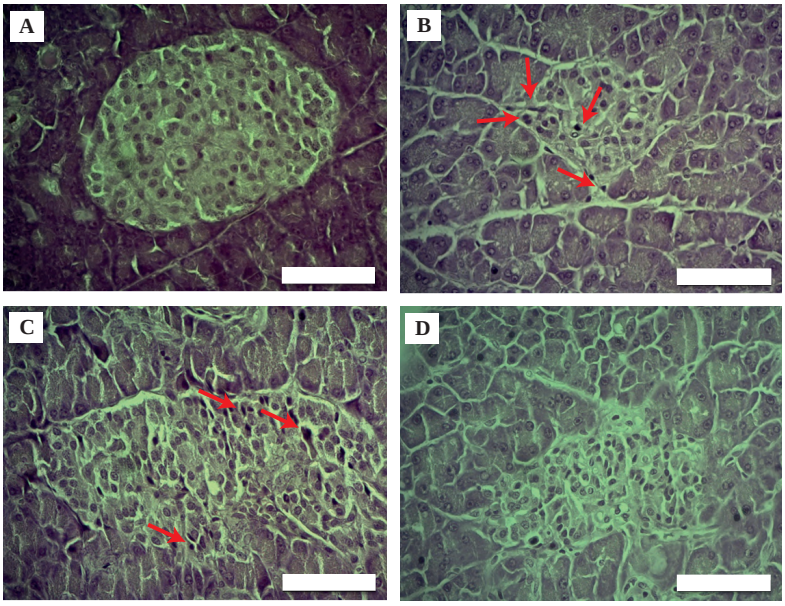

Figure 1. Insulitis in pancreatic islet of Langerhans. A: Control group with insulitis score of 0; B: Group 1 with insulitis score of 3; C: Group 2 with insulitis score of 2; D: Group 3 with insulitis score of 0. Red arrows: Inflammatory cells in the islets of Langerhans. White bar: 100 µm.

Control Group and Group 3 (p=0.046), Group 1 and Group 2 (p=0.04), and the last between Group 1 and Group 3 (p=0.034). However, Group 2 compare with Group 3 showed no significant difference (p=0.131) (Table 2). According to the Mann-Whitney test showed the significantly different insulitis scores between Control Group compared to all treatment groups (Group 1, Group 2, and Group 3), and also between Group 1 compared with Group 2 and Group 3.

Discussion

The increase of FBG levels in all treatment groups after STZ induction was caused by pancreatic damage followed by insulin deficiency. The hyperglycemic state after STZ induction for 6 days causes an insulitis state that continues to be severe for up to 3-4 weeks, and it has been demonstrated in previous study that the STZ-induced hyperglycemic state can last up to 3 months.(16) The STZ mechanism is related to the type-1 DM pathogenesis. There is an imbalance of gut microbiota that causes abnormal modulation of immune cells in gut-associated lymphoid tissue (GALT), which related to pancreatic lymph nodes (PLN) and triggers pancreatic cell damage.(19) Cytotoxic T-cells are the main cause of pancreatic cell damage, followed by macrophages and other immune cells resulting in insulitis and finally insulin deficiency due to cell death enhancement and decreased amount of pancreatic cells.(20,21)

Inflammation which characterized by the accumulation of lymphocytes, a lot of intercellular spaces with smaller nuclei and no clear boundaries, was occured in Group 1 with 75% of the islets of Langerhans. Other study showed degeneration of endocrine cells leading to cell necrosis in DM rats, by the presence of empty spaces in the islets and irregular shape of the cells (polymorphs).(22) The islets of Langerhans of the Group 2 and Group 3 were repaired, which was marked by a decrease of the insulitis degree score to 0 due to the anti-inflammatory mechanism found in the group. The treatment using probiotic *L. acidophilus* decreased TNF-α levels, which block the nuclear factor kappa B (NF-κB) activity. NF-κB becomes activated due to stimulation of reactive oxygen species (ROS) agents causing endothelial dysfunction, DNA destruction, and physical harm.(23) In addition, NF-κB functions in controlling the expression of genes encoding proinflammatory cytokines, like TNF-α, IL-1β, and IL-6. Thus, it is possible that one of the direct mechanisms for this repair that the inhibition of nuclear factor-kappaB (NF-κB) will attenuate the inflammation process of the islets of Langerhans (insulitis).

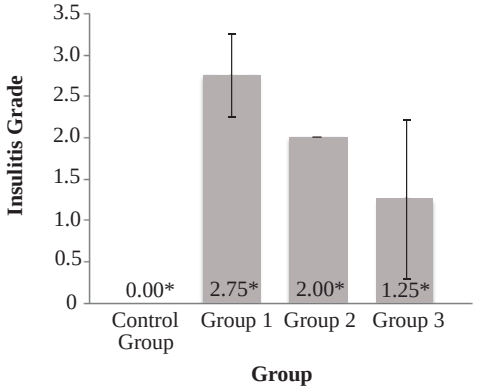

Figure 2. Insulitis grade assessment displayed in the mean score of the grade of insulitis. *p<0.05 is considered as significant, tested with Kruskall Wallis.

Table 2. Comparison of pancreatic histopathology scores in the degree of insulitis.

Group *vs.* Group		*p*- value
Control group	Group 1	0.011*
	Group 2	0.008*
	Group 3	0.046*
Group 1	Group 2	0.040*
	Group 3	0.034*
Group 2	Group 3	0.131

*$p<0.05$ is considered as significant, tested with Mann-Whitney.

(24) *L. acidophilus* also regulates the mRNA expression of toll-like receptor (TLRs) trough the activation of the NF-κB and mitogen-activated protein kinase (MAPK) signaling, which modulate the production of proinflammatory cytokines, thereby reducing the inflammatory response that occurs in insulitis.(25)

Several previous studies had reported that proinflammatory cytokines (TNF-α, IL-1β, and IL-6) significantly increase in type-1 DM, which directly toxic to pancreatic β-cell. These proinflammatory cytokines also stimulate M1 macrophages phenotype and promote the increasing cytokine production mediated more β-cell death. The glucose homeostasis and metabolism indirectly influence by the action of IL-6 on the pancreatic β-cells, while TNF-α directly induces ROS in pancreatic β-cell trough the stimulation of protein kinase C/phosphatidylinositol-3 kinase and MAPK pathways. The pancreatic β-cell has been revealed more susceptible to IL-1-triggered apoptosis thus associated with β-cell failure.(26,27)

Currently, it is known that *L. acidophilus* able to modulate the gut microbiota, thereby increasing SCFAs-mediated activation of nutrient sensor G-Protein Coupled Receptor (GPR)41 and GPR43 in pancreatic islets.(28) The activation of the receptor GPR43 increases the secretion of GLP-1 from intestinal L-cells, which function to stimulate insulin gene transcription, growth islet cells, neogenesis, thereby helping to improve the state of diabetes with pancreatic cell damage, so that *L. acidophilus* has an indirect effect on improving insulitis levels.(10)

In this study, two doses of probiotic were administered in order to compare the effects on pancreas histopathology. The histopathological features of the pancreas in type-1 DM rats treated with $1.5x10^9$ CFU/mL/day showed the average of insulitis grade lower than dosage of $1.5x10^8$ CFU/day. Several previous studies have shown the different clinical results in dosage range from 10^6 to 10^{10} CFU/mL, which was affected by the duration of administration and the specific characteristics of the probiotic strain. The administration of both probiotic strains *L. acidophilus* dose of $7.23x10^6$ CFU/mL and *B. lactis* $6.04x10^6$ CFU/mL for 6 weeks showed the improvement of oxidative stress thereby preventing apoptosis in pancreatic β-cells.(14) Other study showed that STZ-induced diabetic rats treated with *L. brevis* at a dose of 10^9 CFU for 4 weeks had pancreatic histopathological features with repair of the islets of Langerhans.(15) The administration of *L. acidophilus* combination with *Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bifidobacterium breve* also showed the potential effect in the adipose tissue to reduce the inflammation and also insulin signaling improvement. The mixture of several probiotics strain may interfere the type-1 DM development trough controlling the inflammation, prevention of pancreatic β-cell death, increasing the production and expression of IL-10, that is an anti-inflammatory cytokine.(29,30)

Conclusion

The administration of probiotic *L. acidophilus* FNCC 0051 at both dose of $1.5x10^8$ CFU/mL/day and $1.5x10^9$ CFU/mL/day for 21 days in STZ induced type-1 DM rats shows significant improvement on pancreatic histopathological features, with significant decrease of insulitis scores compared to the DM group. The findings of this study could be a fundamental data about the potential benefit of probiotic *L. acidophilus* FNCC 0051, and for the development of further research on cytokines secreted by immune cells that affect the insulitis conditions in type-1 DM.

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Authors Contribution

MS and SRP were involved in planning and supervised the work, TAA and KS performed the measurements, MS, TAA, NDE, EY, and AAN processed the experimental data, performed the analysis, drafted the manuscript and

designed the figures. MS, TAA and NAM performed the xyz calculations and statistical analysis. MS, TAA, and SRP aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

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