Lactobacillus reuteri DSM 17938 Increased the Fibroblast Count in the Wound Healing Process on Diabetic Wistar Rats

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Abstract: ***Scope**: This study aims to see the Lactobacillus reuteri DSM 17938 effect on diabetic Wistar rats' wound healing process. **Method and Results**: The randomized post-test only control group was applied in 28 male diabeticWistar rats aged 3-4 months. The subjectswere dividedinto four groups; two study groups with5 and 12 days intervention durationand two control groups.The study groups were given glimepiride and Lactobacillus reuteri DSM 17938; the control groups were given glimepiride and placebo. All interventions were given for 30 days before wounded using a punch biopsy and given for 5 and 12 days after. Histopathological examination was performed to assess neovascularization, fibroblast count, and epithelialization.On day 5, the study found that the mean fibroblasts count in the study group was higher than the control. However, there were no significant differences in neovascularization, the epithelial thickness andgap between the study and control groups.On day 12, similar results were found, although there wasan improvement in all variables. **Conclusions**: Lactobacillus reuteri DSM 17938 increased the fibroblasts counts on day five but did not improve neovascularization and re-epithelialization in diabeticmale Wistar rats; nevertheless, the probiotic showed a potential positive effect.***

Keywords: diabetes mellitus, re-epithelialization,fibroblast, *Lactobacillus reuteri* DSM 17938, wound healing.

List of Abbreviations:

hs-CRP : *High Sensitivity C-Reactive Protein*
IDF : *International Diabetes Federation*
IFN-y : *Interferon gamma*
Ig : *Immunoglobulin*
IL- : *Interleukin-*
KGF :*Keratinocyte Growth Factor*
LDL : *Low Density Lipoprotein*
MDA : *Malondialdehyde*
MMP : *Matrix Metalloproteinase*
NA : *Nicotinamide*
NF-kB : *Nuclear Factor-kB*
NO : *Nitrit Oxide*
PDGF : *Platelet Derived Growth Factor*
ROS : *Reactive Oxidative Species*
TGF-α : *Transforming Growth Factoralfa*
TGF-B : *Transforming Growth Factor beta*
TLR : *Toll Like Receptor*
TNFα : *Tumor Necrosis Factor*
VEGF : *Vascular Endothelial Growth Factor*

1. Introduction

The World Health Organization (WHO) predicts a continuous increase in diabetes incidence as a global health threat. WHO also predictsthat the number ofdiabetes people in Indonesia was 8.4 million in 2000 and will be around 21.3 million in 2030.[1]Diabetes mellitus is a metabolic disease with various potential complications in the course of the disease. A diabetic ulcer is a chronic complication that often occurs in people with diabetes. Approximately 25% of people with diabetes are at risk of developing diabetic ulcers during their lifetime, and poor wound healing is the initial reason for morbidity and mortality among these patients. Ischemia, neuropathy, and infection are the three main elements that cause complications in diabetic ulcers. In addition to this, hyperglycemia, hyperinsulinemia, dyslipidemia, increased inflammatory markers, and oxidative stress also plays a significant role in diabetic ulcers' pathogenesis so that infection and delayed wound healing complicate therapy in diabetic ulcers.[2]

The rising problem of antimicrobial drug resistance globally makes the idea of taking probiotics interesting and relevant. Probioticshave been studied for its ability to strengthen the immune system, anti-inflammatory effects, and therefore enhance the wound healing process.[3,4] Research by Mohseni shows that probiotics supplementation (*Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus fermentum, Bifidobacterium bifidum*) for 12 weeks in patients with diabetic foot ulcers has beneficial effects on reducing ulcer size, glucose metabolism, total cholesterol, high sensitivity C-reactive protein (hs-CRP), plasma Nitric Oxide (NO), total antioxidant status and Malondialdehyde (MDA).[5]A study by Ming-Chia on *Lactobacillus reuteri* shows its role as an anti-diabetic where *Lactobacillus reuteri* ADR-3 reduces insulin resistance, while *Lactobacillus reuteri* ADR-1 lowers HbA1c levels, cholesterol and increases antioxidant levels in mice fed a high fructose diet (Ming Chia et al., 2018).[6] Mobini et al. were then conducted the first study of the metabolic effects of the probiotic bacteria;*Lactobacillus reuteri* DSM 17938 against type 2 diabetes patients for 12 weeks, which increased insulin sensitivity.[7,8]

Lactobacillus reuteri has previously been studied on the wound healing process by Poutahidis, where the wound healing process in mice consuming *Lactobacillus reuteri* ATCC PTA 6475 in drinking water was significantly faster than the control group.[9] Another study by Varian and Poutahidis using *Lactobacillus reuteri* DSM 17938 also showed that the wound healing process in mouse and human skin was faster than the control group.[10] Besides, research conducted by Khmaladze et al. and Butler et al. underlined the advantages of *Lactobacillus reuteri* DSM 17938, which may benefit general skin health, avoiding UVB-R-mediated inflammatory cascades, preventing premature aging, and increasing skin barrier.[11,12]

Various previous studies have provided evidence that probiotics consumption can accelerate wound healing and have anti-diabetic properties but rarely conducted in a chronic diabetes condition in the animal study. Hence,this study aimed to see the effect of giving *Lactobacillus reuteri* DSM 17938 probiotic in increasing neovascularization, fibroblast cell count, andre-epithelialization of the wound healing process of male Wistar with diabetes mellitus.

2. Experimental Section

A randomized post-test only control-group design was conductedin 3 to 4-month-old male Wistar rats (*Rattus norvegicus)*. The simple random technique was applied to the appropriate Wistar rats population. Diabetesmale ratsaged 3 to 4 months and had approximate weights of 150-200 grams were the study's inclusion criteria.

The sample size computed accordingly using the Federer formula with an additional 10% drop out, counting a total of twenty-eight male Wistar rats needed in the study. The total sample was then divided evenly into four groups—Two groups assigned as study groups with differentduration of the intervention, namely 5 and 12 days, and two groups as control groups.

The study lasted for approximately two months, including animal preparation. Male Wistar rats (*Rattus norvegicus*) aged 3-4 months with a weight of 150-200 grams are given Nicotinamide 230 mg/kg BW injection and an intraperitoneal injectionStreptozotocin with a single dose of 65 mg/kg BW. On the 7th day after injection, the subject's fasting blood glucose levels measured by glucometer, the level of >135 mg/dL were determined to be diabetic mice.

The probiotic bacteria *Lactobacillus reuteri* DSM 17938 in tablet (108 CFU) with the trademark *Interlac*was used in this study.The dose to be given is 2.5 x 10^6 CFU/day per 200 grams of subject body weight obtained by converting the research dose by Varian et al. (2017) using the Harmita (2008) conversion table.All study subjects were given glimepiride(0.0504 mg/200 gr BW)orally once a day for 30 days before wounded using a punch biopsy.The twostudy groups were also given *Lactobacillus reuteri* DSM 17938 (2.5x10^6 CFU/200 gr BW)orally once a day; meanwhile, the two control groups were given distilled water once a day for 30 days before wounded, and continuously being administered for the next 5 and 12 days.The wound was made with a punch biopsy, 3 mm in diameter, parallel to the vertebrae, 5 cm from the ear.

On the 5th day after the injury, one study and one control group euthanized, and tissue cutting was performed using the ellipse excision technique, which included the wound bed tissue and histological preparations with HE staining.A similar procedure applied for the 12th day after the injury.The examination of neovascularization, fibroblast cells count, and re-epithelization were performed on days 5 and 12 after the injury. The neovascularization measurement was carried out by looking at the increase in new blood vessels' formation using a 40 times magnification objective lens. An improvement in wound tissue regeneration, seen from the significant difference in the number of new vessels in the study group compared to the control. The fibroblast cells countexamination was also conducted usingan objective lens at 40 times magnification, an improvement in wound tissue regeneration, seen from the significant difference in the number of fibroblasts in the study groups compared to controls. Re-epithelialization is assessed by measuring the thickness of the epithelium (thickness of the new epidermal layer formed in the wound area) histomorphometrically with micrometers (μm), and also measures the distance of the epithelial gap from the edge. The wound was then observed and measured using a CX21 microscope connected to an Optilab viewer 2.2, with a four times magnification objective lens. The images were processed with Image Raster 3 software, then measured histomorphometrically in micrometer (μm) units. Reepithelialization was indicated by increasing epithelial thickness and closure of the epithelium wound gap in the study group compared with controls.

All the procedures in this study approved by the local ethical committee for animal study, Udayana University, with ethical clearance number1071/UN14.2.2.VII.14/LT/2020. The statisticalanalysis has utilized SPSS Ver. 17 for windows. Univariate analysis is composed of descriptive analysis, the test of data normality, and homogeneity test. The independent t-test was carried out for comparing both groups.

3. Results

In examining and calculating variables, identifying epithelial thickness and gaps could not be carried out on two observations on the 5th day due to the tissue preparation process. The epithelial thickness and gap measurements (epithelialization) could not be identified in one sample in the control group and one sample in the treatment group. The total observation on the epithelialization variable on day five only used six samples each. The results of the descriptive analysis of each group are presented in table 1.

[Insert Table 1 here]

The descriptive results show that the mean number of neovascularization per field of view on day 5 was lower in the study group. On the contrary, on day 12, the study group's mean was higher than the control group. The mean number of fibroblasts per field of view on day five and day 12 was higher in the study group than the control group. The mean epithelial thickness (µm) on day 5 was higher in the study group, but on day 12, the mean epithelial thickness was lower in the study group than in the control group. The mean epithelial gap (µm) on day 5 was more expansive in the study group but narrower at day 12 compared to the control group.

A comparability test on the variables of neovascularization, fibroblasts, epithelial thickness, and the epithelial gap was carried out to compare the mean of each variable in the control and treatment groups at two different observation times. Analysis of significance was tested using the Independent Sample T-Test on the variables of neovascularization, fibroblasts, and epithelial thickness. The Mann Whitney U test was used in the comparison of the epithelial gap. The results of the comparability test between groups are presented in table 2.
[Insert Table 2 here]

4. Discussion

The results showed an increase in the mean number of fibroblasts and epithelial thickness on day five and decreased vascular formation around the study group's woundthan the control. This shows an immunomodulatory effect that works from the administration of *Lactobacillus reuteri* DSM 19738 in DM conditions.[7,13] The proliferation phase characteristics with cellular indicators, the number of fibroblasts, and better epithelial thickness were found on day five compared to control. These results are in line with research by Bermudez-Brito, which states that *Lactobacillus reuteri* has benefits in terms of accelerating the wound healing process.[14]

In contrast to the results found on day 12, the median epithelial gap was still better in the control group than the study group, and cellular indicators did not show the expected results. The normal proliferation phase was assumed to have occurred, but fibroblast activity was still higherthan the control group and lower epithelial thickness, although the epithelial thickness increased compared to day five in the study group. This suggests that other possible factors may influence the wound healing process. On dayfive, themeannumberoffibroblasts in thestudygroupis significantlyhigher thanthecontrolgroup. Meanwhile, on the 12th day of observation, there was no significant difference.

The mechanism of action involved is that the metabolite results from *Lactobacillus reuteri*can modulate the innate immune system by interacting with host cells such as neutrophils, short-chain fatty acids produced by Lactobacillus bacteria. This modulation can suppress the nF-KB pathway in neutrophil cells, reducing NO formation and stress oxidative increases antioxidants, which indirectly reduce inflammatory activity. In macrophage cells, the metabolites of *Lactobacillus reuteri*also stimulate macrophage (M2) cells to secrete anti-inflammatory cytokines and secrete growth factors that can stimulate fibroblast proliferation, secrete collagen, form new blood vessels, and form granulation tissue that will aid wound closure.[8,14,15]

The results of the observation of fibroblasts on day five are in line with research conducted by Poutahidis and Varian. In their study, the mice given *Lactobacillus reuteri* experienced a decrease in neutrophils, an increase in fibroblasts, and re-epithelialization on the 6th day, an early sign of the proliferation phase that has taken place.[9,10] In line with Nannan Han research, in the mice given *Lactobacillus reuteri* injection in the treatment group, wound healing and increased fibroblasts were seen on days 5 and 7 compared to the control group.[16]

On the 5th day of observation, the mean number of neovascularization in the study group showed that the lower mean difference was not significant by 24% of the control group. On day 12, the mean number of neovascularization in the study group showed a higher mean difference, which was not significant by 3% from the control group. The 12th days' observations showed that neovascularization, which was expected to have decreased, was still increasing. These results were different from the research conducted by Poutahidis, where the administration of *Lactobacillus reuteri* significantly accelerated wound contraction in mice on the 6th day after the injury and marked by increased vascular formation.[9] However, this study was conducted on mice without DM disorders, which probably underlies the differences. The vascular formation disorder in the subject may occur due to angiopathy in DM conditions that cannot be resolved by probiotics in the study.

Endothelial cell dysfunction is associated with decreased angiogenesis in diabetic wounds, where previous studies showed that VEGF-A protein and mRNA levels in diabetic mice wounds decreased significantly compared to normal healthy control mice.[17] In Nannan Han's previous study, the injection of *Lactobacillus reuteri* showed the acceleration of wound healing through the TGF-B1 growth factor activation pathway. Although this growth factor affects fibroblast proliferation, its role in promoting angiogenesis binds to the VEGF growth factor, wherein diabetes, the VEGF level decreases, and there is a possibility that a small number of VEGF receptors or defects in the VEGF receptor are unable to help TGF-B1 in inducing the process. angiogenesis.[2,10,16]Examination of wound healing parameters in this study focused on histological examination of fibroblast tissue, neovascularization and epithelialization with hematoxylin and eosin staining to see cells that played a role in wound healing and did not perform immunohistochemical examinations to determine growth factors, cytokines, and Treg cells that play a role in the healing wound. The assumption of hyperglycemia and chronic inflammation that may occur can explain the dysfunction and impaired recruitment of endothelial progenitor cells from the bone marrow, which disrupts blood vessel regeneration.[18–20]

On the 5th day of observation, the mean epithelial thickness and the treatment group's comparability test showed an insignificant increase by 4% of the control group. The median epithelial gap and the study group's comparability test showed no significant median widening with the control group. On the 12th day of observation, the mean epithelial thickness in the treatment group showed a lower mean difference, which was not significant by 22% of the control group. However, both observation times showed an increase in epithelial thickness and epithelial gap reduction in the study group. These results are different from the research conducted by Poutahidis et al., Where the administration of *Lactobacillus reuteri* significantly reduced the wound gap (re-epithelialization) in mice after injury.[9]The anti-inflammatory modulation effect of *Lactobacillus reuteri* is to activate the TGF-B growth factor that inhibits proteolytic enzymes and stimulates various chemotaxis that can increase epithelialization stimulation of migration and proliferation of keratinocytes.Proliferation phase that extends in DM conditions, where granulation is formed first at the base of the wound, then fills in the gaps and epithelialization caused by a lack of oxygen supply,thus trigger macrophage activity in the tissue. The epithelialization of the wound tissue is disturbed by the migration of keratinocytes, which will form the outer protective layer or stratum corneum so that the wound moisture decreases and results in slower wound healing. [21–23]

The longer duration of probiotic administration and DM conditions in this study may act as factors that influence significant differences compared to other studies. The study by Campos showed that the acceleration of wound healing was marked by a significant increase in the number of vascular, fibroblasts, and epithelialization on day 10 in the treatment group compared to the control group. Campos also stated that Wistar rats' blood sugar condition in his study did not show a significant correlation with the narrowing of wounds in The treatment group that received probiotic therapy. However, the DM condition in that study was acute after 72 hours of Alloxan injection. Meanwhile, this study's DM condition occurred much longer and is likely to describe DM's chronic condition, even though glimepiride therapy has been received. [24]

This study shows that DM conditions in male Wistar rats can affect wound healing. The administration of higher doses, longer duration of administration, and topical *Lactobacillus reuteri* preparations could be considered in future studies. Research conducted by Khodaii et al. demonstrated the effectiveness of accelerating wound healing of *Lactobacillus reuteri* in an ointment applied to wounds on Wistar rats', although it was not specific to DM conditions or chronic wounds.[25]Besides, the evidence of multi-strain combination lactobacillus also shows a more significant difference in wound healing of DM patients than giving one type of strain because of the more specific inter-cellular mechanism of action in each strain.[5,7]

Lactobacillus reuteri is found in several parts of the human body, such as in the digestive tract, bladder, skin, and breast milk. The role of *Lactobacillus reuteri* in the immune system is to involve several cells such as neutrophil cells, macrophages, and T cells called Treg cells to increase anti-inflammatory production and decrease pro-inflammatory reactions. This proves that Lactobacillus reuteri can affect immune regulation and exhibits anti-inflammatory effects that can inhibit the skin's aging process.

The expected effect of giving *Lactobacillus reuteri* probiotic in this study is to help accelerate the wound healing process in diabetic male Wistar rats compared to controls. The results showed that the immunomodulatory and anti-inflammatory effects of *Lactobacillus reuteri* were shown to increase the number of fibroblasts in the treatment group compared to the control group on day five. However,it is challenging to prove other cellular indicators that encourage further research regarding the effect of probiotics on wound healing by selecting different indicators such as immunohistochemistry, optimization of dosage, and administration of *Lactobacillus reuteri* probiotics, and considering the optimal duration of intervention.The study also strongly suggests exploring the effect of *Lactobacillus reuteri* on insuline sensitivity. This research is expected to increase research evidence on the potential of *Lactobacillus reuteri* probiotics for wound healing in experimental animals suffering from diabetes.

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Author Contributions

KeziaNatania, Ida Sri Iswari and Ni WayanWinartiled the methode and experimental design. KeziaNataniaand Ida Sri Iswariwere the principal investigators during the experiment.KeziaNatania, Ida Sri Iswariand Ni WayanWinarti performed data management and analysis. KeziaNataniawrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Statement

The author(s) declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article

Tables

Table 1: Descriptive Statistics for Day 5 and 12 after Injury in All Groups

Observation Days	Variable	Group	Freq.	Mean±SD	Median	Min	Max
Day 5th	Neovascularization	Control	7	6,8 ± 4,4	6,33	1	14,7
		Study	7	5,1 ± 1,6	5,67	2	6,3
	Fibroblast Counts	Control	7	24,6 ± 13,7	22,67	3	38,3
		Study	7	56,7 ± 12,3	54,33	42,3	79
	Epithelial Thickness (µm)	Control	6	64,7 ± 45,2	46,5	18	129,3
		Study	6	65,3 ± 17,0	69,17	43	84,7
	Epithelial Gap (µm)	Control	6	406 ± 706	0	0	1725
		Study	6	624 ± 674	534	0	1813
Day 12th	Neovascularization	Control	7	6,0 ± 2,3	5	3,7	10,3
		Study	7	6,2 ± 4,8	4	1	15,3
	Fibroblast Counts	Control	7	45,6 ± 13,0	48,67	26,3	63
		Study	7	60,8 ± 16,5	67,67	39,3	79
	Epithelial Thickness (µm)	Control	7	87,4 ± 36,0	69	57,7	156
		Study	7	68,5 ± 23,8	71,67	24	92
	EpithelialGap (µm)	Control	7	675 ± 749	164	0	1522
		Study	7	510 ± 629	394	0	1397

Table 2: Comparative Analysis of Wound Healing Process Between Study and Control Groups on Day 5 and 12 after Injury

Observation Days	Variable	Freq.	Group		p-value
			Control	Study	
Day 5th	Neovascularization (Mean±SD)	7	6,8±4,4	5,1±1,6	0,366*
	Fibroblast Counts (Mean±SD)	7	24,6±13,7	56,7±12,3	0,001*
	Epithelial l Thickness (Mean±SD)	6	64,7±45,2	65,3±17,0	0,976*
	Epithelial Gap (Median(min-max))	6	0(0-1725)	534(0-1813)	0,393**
Day 12th	Neovascularization (Mean±SD)	7	6,0±2,3	6,2 ± 4,8	0,907*
	Fibroblast Counts (Mean±SD)	7	45,6±13,0	60,8 ± 16,5	0,079*
	Epithelial l Thickness (Mean±SD)	7	87,4±36,0	68,5 ± 23,8	0,267*
	Epithelial Gap (Median(min-max))	7	164(0-1522)	394(0-1397)	0,506**