

Lactobacillus plantarum modulatory Effect on the Secretion of Interleukin-10, TGF β , and *Fibronectin* in Macrophages and Skin Dermal Fibroblasts Culture

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Abstract: Impaired wound healing in diabetic patients is associated with increased pro-inflammatory cytokine TNF α and IL1 β , as well as the reduction of inflammatory cytokines IL10 and TGF β . *Lactobacillus plantarum* is known to modulate the immune response towards the regulatory cytokine that increases anti-inflammatory cytokines. The purpose of this study is to determine the effect of *L. plantarum* on the secretion of IL10, TGF β , and fibronectin of macrophages and dermal fibroblasts. Research using cell culture of peritoneal macrophages and dermal fibroblasts of Wistar rats, cultivated in RPMI + 10% FCS, and stored in a 5% CO₂ incubator at 37°C. *S. aureus* atcc 25923 in Mueller Hinton and *L. plantarum* atcc 8014 in MRS were harvested at mid-log phase and then the suspension was made at doses of 10⁶, 10⁷, 10⁸ cells/mL and subsequently heat-killed. Macrophage cultures were exposed to *S. aureus* for 6 hours washed and then treated by combination of *S. aureus-L. plantarum* (SALP) and *L. plantarum* for 24 hours. Next the supernatant was separated, added into the culture of fibroblast cells for 24 hours prior to measurement of IL10, TGF β , and fibronectin levels by ELISA. Analysis using ANOVA and Tukey's tests with $\alpha = 5\%$ shows the results where overall exposure of *L. plantarum* along with increasing doses had increased the levels of TGF β macrophages and fibronectin fibroblasts, meanwhile SALP exposure increased the lowest level of fibronectin. Increased level of IL10 fibroblasts was reached only on the highest dose of LP exposure. Thus, it was concluded that *L. plantarum* increased the levels of TGF β and fibronectin ($p < 0.000$).

Keywords: *L. plantarum*, IL10, TGF β , fibronectin, immunomodulator

1. Introduction

Sedentary lifestyle in urban communities currently allows an increase in a variety of metabolic diseases such as diabetes mellitus (DM) that affects various organs and can destroy the lives of patients due to serious complications, one of which is the ability to heal chronic wounds, known as diabetic ulcers.

Impaired wound healing process is a complication of diabetes and it can lead to lower limb amputations [1],[2],[3]. In addition, an increased incidence of complications due to wounds in diabetic patients who underwent surgery, will increase risk by metabolic disorders associated with DM [4]. Wound healing is a complex pathophysiological process that is affected by many factors and it consists of a complex process to restore tissue integrity. This process is mediated by a variety of cells, cytokines, matrix, and growth factor. The coordinated interaction between cellular and acellular components of wound healing process is very important, dysregulation of these factors can exacerbate tissue inflammation and trigger more severe illness that cause complications or abnormal wound healing termed as diabetic ulcers [2].

In diabetic ulcers, there is multiple impaired wound healing in the form of chronic inflammatory state that occurs primarily due to imbalance of pro-inflammatory and anti-inflammatory cytokines. Therefore, inflammation recovery is very important to the success of wound healing. Wound inflammation is enhanced through various mediators that are supposed to be very controlled. Macrophage cells existing in

the injured area are the key to the inflammation process. There is a decrease of macrophage functions, including phagocytosis activity, and high production of pro-inflammatory cytokines tumor necrosis factor alpha-TNF α and interleukin-1-beta-IL1 β , as well as low secretion of interleukin-10-IL10 and transforming growth factor-beta-TGF β . However, the factors underlying chronic inflammation in diabetic ulcers need to be studied further [3],[4].

Infiltration of inflammatory cells in the injured area plays an important role in the outcome of cellular processes and the quality of wound healing response. Manipulation of the number and activity of inflammatory cells in the injured area can be used as an approach to control the wound healing process. Various cytokines produced by the immune cells bring a profound effect on fibroblast migration, proliferation, and production of extracellular matrix. Previous in vitro studies showed that interaction between inflammatory cells and fibroblasts can modulate several fibroblast functions, including the production of collagen and fibronectin [5],[6]. The probiotic *Lactobacilli* has been proven to give many health benefits through inhibition of pathogenic bacteria colonies, restoration of microbial homeostasis through interaction of microbes, improvement of epithelial barrier function, and modulation of immune response. Studies on immune-modulatory effects of probiotics in the gastrointestinal tract have proven [7],[8].

Based on several studies on the effects of probiotics on inflammation and dermal wound healing, such as by Huseini

et al showed that *Lactobacillus plantarum* plays a role in the early stages of wound healing process, by rapidly reducing inflammation and improving epithelialization [9]. Today, many methods include medication, pressured therapy, skin bioengineered, tissue substitutes, hyperbaric oxygen therapy, surgery, etc. have been performed for treatment of diabetic ulcers. However, because the success rate has not been satisfactory, there is still a great challenge for researchers to find appropriate methods in treating diabetic ulcers [4],[10].

Several studies have shown the effect of probiotics on wound, however the mechanism on how probiotics can be involved in prevention and treatment of some pathological states, including in the process of wound healing in diabetic patients, is still not clearly revealed. Effect of the probiotic *Lactobacilli* on the secretion of TGF β , IL10, and *fibronectin* by macrophages and fibroblasts are still needed to be researched to define and explain the changes in the secretion of TGF β , IL10, and *fibronectin*, as indicators or predictors of diabetic ulcer healing.

2. Material and Methods

2.1 Conceptual Framework

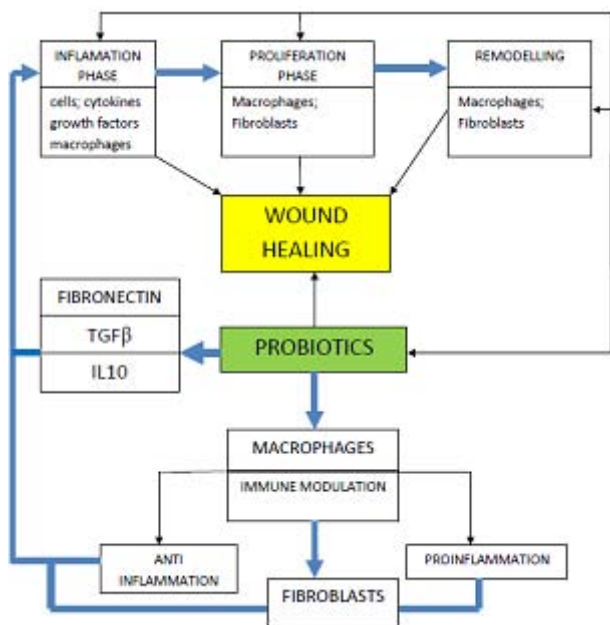


Figure 1: Framework Concept

2.2 *Staphylococcus aureus* and *L. plantarum* cultures

S.aureus-SA culture was obtained from atcc 25923, cultured in Mueller Hinton, and incubated at 37°C for 24 hours under aerobic condition. *L.plantarum*-LP that was obtained from atcc 8014 was cultured in MRS and incubated at 37°C for 24 hours under anaerobic condition. Those cultures were harvested in mid-log phase and bacterial suspension was then made by comparing it against McFarland standard and adjusted up to 10⁶,10⁷,10⁸ cells/mL (*S.aureus*) and 10⁶,10⁷,10⁸ cells/mL (*L.plantarum*), which were confirmed with a spectrophotometer. Next, the cultures were heat-killed by keeping it in a water bath at 70°C for 30 min and leaving it at ambient temperature prior to storing it at 4°C until used [11].

2.3 Culture of peritoneal macrophages

Macrophage cells were obtained from *Wistar* rats, isolated and cultured as described by Freshney et al, with minor modification. Briefly, macrophages washed three times. The cells were counted and their viability was assessed by *Trypan* blue. Next, macrophages were cultured in RPMI + 3% FCS in a 5% CO₂ incubator at a temperature of 37°C for 45 min, so that the macrophages attached themselves to the base of the plate. Treatment was started after 4-24 hours post plating [12].

2.4 Stimulation of macrophage cells with *S.aureus* and treatment of *L.plantarum*

Peritoneal macrophage cell culture that had been incubated overnight was then treated with SA at doses of 10⁶,10⁷,10⁸ cells/well for 6 h. The supernatant was removed and washed with RPMI 2 times, then LP was added with doses of 10⁶,10⁷,10⁸ cells/mL. Sample was then incubated for 24 hours and the supernatant was transferred into an ELISA plate to measure its macrophages and cytokine levels [12].

2.5 Cultures of dermal fibroblasts

Samples of dermal tissue were sliced in the size of 2 – 3 mm², transferred to a plate containing DMEM + 10% FCS, and stored in a 5% CO₂ incubator at 37°C. The third passage of fibroblast cells whose viability had been assessed was then ready to undergo treatments using the supernatant of the macrophages culture for 24 h and then the supernatant was separated for measurement of fibroblasts cytokine levels [12].

3. Results

Based on the results of this research, it was found that peritoneal macrophage cells and dermal fibroblasts cells exposed to *L.plantarum* and the mixture of *L.plantarum* and *S. aureus* (SALP) for 24 h did not increase secretion of IL10 significantly (p>0.05). Figure 2 shows that although it is not statistically significant, LP exposure to macrophages at the highest dose seems to increase the secretion of IL10 fibroblasts compared to SALP exposure at all doses and LP exposure at low doses. These results are not in line with the results reported by Kaji, et al in which LP treatment at the highest dose of 30 mg/mL for 24 h was able to produce the highest production of IL10 macrophages [13]. However, results of the current study are in accordance with previous studies performed on human cell cultures, where the secretion of IL10 plasma was not detectable after exposure to LP for 24 h [14]. After exposure to LP for 24 h, secretion of TGF β macrophages and *fibronectin* fibroblasts were significantly increased (p<0.000). The highest increase of *fibronectin* secretion was achieved through LP exposure at a dose of 10⁷ cells/mL, however, at higher dose of 10⁸ cells/mL *fibronectin* secretion decreased. These results are in accordance with previous studies that stated that *Lactobacillus spp* exposure can increase secretion of interleukin-10 [15], other studies have also stated that

exposure to *S. aureus* bacteria increases the secretion of *fibronectin* by dermal fibroblasts [16],[17].

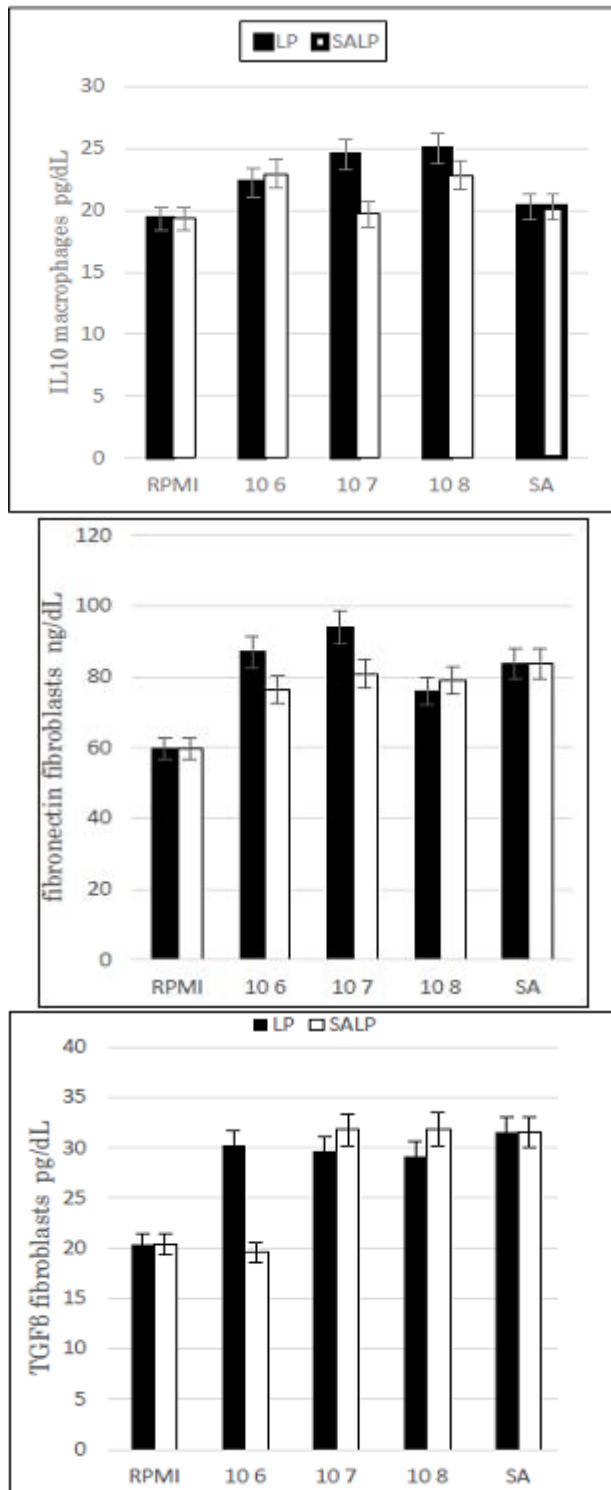


Figure 2: Effects of SALP and LP treatment on macrophage cells and fibroblasts culture of different cytokines in rats. The cytokine levels determined by Elisa for TGFβ, IL10, and fibronectin from RPMI as control, *L.plantarum*-LP, *S.aureus*-*L.plantarum*-SALP, and *S.aureus*-SA. Data shown are the means ± SD of 3 animals. P values for all significant comparison ($p < 0.05$) are represented.

4. Discussion

Interleukin-10 (IL10) is a cytokine that attracts much attention due to its immune-regulator effects. This cytokine is produced by a variety of immune cells, mainly by macrophages as the major source of IL10, and they are instrumental in the inhibition of inflammatory response as well as in the regulation of differentiation and proliferation of various immune cells. Regulation of IL10 secretion by macrophages is stimulated by various factors such as presence of an endotoxin known as *toll-like receptor*-TLR4-*nuclear factor kappa-light-chain-enhancer of activated B cells*-NFκB [18], and TNFα, as well as an increase in *cyclic adenosine monophosphat*-cAMP and *catecholamines* [19],[20]. Endotoxin can also increase expression of IL10 receptors in fibroblasts [20], as shown in our research. Bacterial endotoxin triggers the release of *catecholamines* that cause upregulation of IL10 by macrophages, mainly in the liver. However, since the current research was designed as an *in vitro* study, there was no interaction between *catecholamines* and liver that can induce the secretion of IL10 by macrophages.

Macrophages and lymphocytes are the primary target of IL10, which can reduce the production of pro-inflammatory cytokines and decrease the ability of an antigen presentation by macrophages and dendritic cells. Macrophages located at the wound site plays an important role in supporting the inflammatory phase of normal wound healing. Macrophages are very important in cleaning a variety of cells and dead tissue in the wound area. Less optimal number and function of macrophages during inflammatory phase will result in slow process of wound healing. Hyperglycemia in diabetes has effect on macrophages function including its *fagocytosis* activity. Macrophages derived from wounds in diabetic mice showed disorder in *sferosistosis*, which causes the accumulation of apoptosis cells, as there was an increase in expression of pro-inflammatory cytokines [3],[21].

Some researchers found that *exopolysaccharide*-EPS derived from macrophages *L. reuterii* increased the secretion of IL10 [22], and *lipoteichoic acid*-LTA derived from *L.plantarum* is able to restore the secretion of IL10 pro-inflammatory into IL12 dominant due to activation macrophage [13]. However, in our study, the effect of *L.plantarum* gave no significant difference ($p > 0.05$) on the secretion of IL10 macrophages and fibroblasts, as shown in Figure 2. Meanwhile, all LP treatment can significantly increase the level of *fibronectin* ($p < 0.000$). High blood glucose level stimulates macrophage cells to increase production of pro-inflammatory cytokines such as *interferon alpha*-IFNα, TNFα, and IL1β. Macrophages isolated from diabetic mice are often infiltrated by high amount of inflammatory macrophage-M1 resulting in interference that causes slow wound healing. An increase in the secretion of IL10 did not occur in this study, which is important to extend the M2 macrophage phase that is a high source IL10, capable to support various aspects of wound repair, including *chemotaxis*, wound contraction, angiogenesis, *re-epithelization*, and regeneration of connective tissue [2]. The Increased level of IL10 was not statistically significant in our study, most probably due to the

temperature of 70°C used in the process of heat-killing was not high enough to produce low-molecular-weight from *L.plantarum* that is able to increase the secretion of IL10, capable to produce high amount of EPS-*L.plantarum* so that high secretion of IL10 can be stimulated.

Transforming Growth Factor- β . Dis-regulation of the TGF β and depletion of the number of macrophages during inflammatory phase of wound healing lead to impaired healing process that ends in chronic wound and diabetic ulcers [23]. These phenomena can be caused by various factors that occur in diabetic patients, where lipid metabolism disorders and prolonged hyperglycemia result in extension of inflammatory phase by high pro-inflammatory cytokines, such as TNF α and IL1 β , accompanied by a decrease in the anti-inflammatory cytokine TGF β and IL10 due to a disturbance in polarization of the macrophages M1 to M2c [1].

Peritoneal macrophages reacted with 10^8 *L.plantarum* successfully increased secretion of TGF β compared to control sample. Supernatant containing TGF β macrophages was able to significantly stimulate the secretion of TGF β fibroblasts ($p < 0.05$), as shown in Figure 2. Increased level of TGF β macrophages was possibly due to suppressive action of the cytosine-phosphat-guanosine-CpG DNA of *L. plantarum* as commensal bacteria. Suppressive action of CpG DNA of *Lactobacilli* is potentially immune-regulatory that inhibits activation of dendritic cell-DC and maintains *T Lymphocyte regulator*-Treg conversion during inflammation, both *in vitro* and *in vivo*. The immunosuppressive ability of CpG DNA of *Lactobacilli* is dependent on its ability to form a complex and prevent phosphorylation of STAT [24]. Yang et al, in 2012 found that *L. acidophilus* was able to down-regulate the Smad7 production to restore the activity of TGF β 1/Smad pathway and improve inflammation. Smad7 production can be induced through the STAT1-dependent Smad7 [25]. Thus, the suppressive ability of CpG DNA of *L.plantarum* can form complex structures with STAT1 that stop STAT1-dependent Smad7 pathway restore activation of TGF β . The increased level of TGF β fibroblasts found in this study showed that TGF β secreted by macrophages can stimulate the secretion of TGF β by fibroblasts. These finding supports the results of several previous studies that stated that TGF β macrophages can stimulate proliferation and differentiation [26] as well as induce the function of fibroblasts in secreting TGF β and *fibronectin* [6],[26],[27].

Fibronectin. Fibroblasts are the main producer of *fibronectin* in a cell. Fibroblasts found in the wound area of diabetic patients showed a decrease in migration and proliferation and an increases in apoptosis. Transformation of fibroblasts into myofibroblast is mediated by a variety of factors including *fibronectin* [1],[2]. Chronic ulcers caused by diabetes decrease secretion of TGF β and IL10 and reduce the transformation of fibroblasts into myofibroblast [3],[4]. The highest increase in *fibronectin* level was caused by intermediate dose of LP exposure as shown in Figure 2. This phenomenon is most probably due to colonization of *L.plantarum* that enhances the production of antigen by macrophages, which further increases the pro-inflammatory cytokines, such as IL4 and anti-inflammatory cytokines, such as TGF β caused by high microbial dose (10^7 cells/dL). IL4

and TGF β have broad functions on fibroblasts, promoting the synthesis of *fibronectin* [27],[28].

Exposure of high dose LP in macrophages cell culture appears to reduce the level of *fibronectin* produced by fibroblasts, most likely to be caused by an increase in the anti-inflammatory cytokines TGF β and IL10, resulting in down regulation cytokines level that leads to a decrease in immune activity causing decreased release of pro-inflammatory cytokines that leads to slower inflammation process so that production of *fibronectin* by fibroblasts is reduced. Moreover, the decreased level of *fibronectin* could also be caused by the pathogenic nature of *L. plantarum* that is far lower than that of *S.aureus*. Pathogenicity of a microbial antigen is caused by several factors including the 'strangeness' of an antigen, the more strange an antigen is, the more the immune response is activated leading to higher level of cytokines released [29], Strangeness of an antigen molecule can be due to many factors, one of them is *unmethylated* CpG DNA that is often found in bacterial DNA [30]. The genus *Lactobacilli* has low CpG, meaning that they have low content of CpG, thus enabling them to slower the immune system activation compared to pathogenic bacteria such as *S. aureus*. The slower the macrophage activation, the lower the production and secretion of pro-inflammatory cytokines are, leading to low level of *fibronectin* secretion [6],[31].

Exposure of SALP combination increased the level of *fibronectin* fibroblasts, but when compared with the increase in *fibronectin* resulted from SA and LP, SALP produced the lowest increase. There is most likely a mechanism that allows inhibition of immune system activation by anti-inflammatory cytokines or a new signaling pathway that can suppress the production of *fibronectin* caused by both microbes. Researchers have written that *Lactobacilli* can stimulate secretion of inflammatory cytokines, such as TGF β and IL10, which in turn lower the activity of pro-inflammatory immune response and decrease the expression of IL4, leading to increased production of *fibronectin* by fibroblasts.

***L. plantarum*, cytokines, and diabetic ulcers.** Patients with diabetic ulcers experience impaired cell function, inflammatory imbalance, impaired proteases, cytokines and growth factors function. There is an increase in fibroblast apoptosis, a decrease in fibroblast proliferation, and an elongation of inflammatory phase due to pro-inflammatory cytokines that suppress secretion of inflammatory cytokine, such as TGF β , IL10, and decrease *fibronectin* level. In this study, it was found that LP exposure was able to modulate secretion of TGF β and IL10 cytokines and increase the level of fibroblast *fibronectin*, which gains new hopes for further *in vivo* research concerning the application of *L.plantarum* to accelerate wound healing process in patients with diabetic ulcers.

5. Conclusion

Based on the results collected, it can be concluded that *L.plantarum* exposure increases the secretion of TGF β macrophages and *fibronectin* fibroblasts, however, only high

doses of *L.plantarum* that are able to increase the secretion of IL10 fibroblasts.

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