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The Anti-inflammatory Activity of Probiotic *Dadiah* to Activate Sirtuin-1 in Inhibiting Diabetic Nephropathy Progression

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Abstract

Purpose: The activation of SIRT-1 in the kidney has become a new therapeutic target to increase resistance to many causal factors in DN development. Furthermore, antioxidative stress and anti-inflammation are essential to preventing renal fibrosis in DN. Therefore, finding "probiotic products" to treat and prevent DN is necessary. This study aimed to analyze the anti-inflammatory of probiotic *dadiah* to activate SIRT-1 in inhibiting DN progression.
Methods: This study is an experimental group designed with a post-test-only control group to observe the effect of *dadiah*, LAB, and bacteriocin on alloxan-induced nephropathy diabetic rats through two control groups and five intervention groups for eight weeks. The expression of antibodies SIRT-1 and TNF-α was examined using Immunohistochemistry and histopathology of kidney tissue. All data were analyzed using ANOVA test.
Results: The treatment of *dadiah*, lactic acid bacteria, and bacteriocin showed a higher expression of Sirtuin-1 than the positive control. They also, reduce TNF-α expression varies significantly between treatments. The highest average of interstitial fibrosis in the C+ groups was substantially different from all groups, but all treatments showed decreased kidney fibrosis. Although all treatments showed a decrease in interstitial kidney fibrosis found in the control group, the treatment using *dadiah* showed the highest result.
Conclusions: *Dadiah* has the potential to the prevention of fibrosis on kidney tissue of alloxan-induced nephropathy diabetic rats. The findings could be to develop novel treatments for DN that aim to reduce the cascade of oxidative stress and inflammatory signals in kidney tissue.

Keywords: *Dadiah*; Sirtuin-1; TNF-α; Diabetic Nephropathy

1. Introduction

Diabetes mellitus (DM) is one of the most significant health problems worldwide. According to the projections, the number of adult diabetic patients will exceed 430 million in 2030. Diabetic nephropathy (DN) is one of the most microvascular complications and is now the leading cause of end-stage renal disease (ESRD) [1-4].The prevalence of DM is increasing and is an essential cause of microvascular diseases such as DN [5]. DN is a serious microvascular complication of DM, and according to data in the United States, it is estimated to be suffered by 44% (30 - 40%) DM patients [3].

The main criteria to diagnose DN is the presence of an increased urinary albumin excretion (UAE), which is divided into microalbuminuria and macroalbuminuria, which is associated with an increased risk of decline in glomerular filtration rate (GFR) and a high risk of kidney failure [6]. Natural-history studies show the occurrence of proteinuria, eventually develops in 30-50% of diabetic persons [7,8]. Many pathways involving DN, such as hyperglycaemia, oxidative stress (OS), and protein kinase C (PKC) activation, have been postulated. As a significant mediator for DN development and progression, the upregulation of AGE receptors (RAGE) [9]. Renal fibrosis, characterized by extracellular matrix (ECM) protein accumulation, leads to CKD, including DN. It found that the process of signalling transformation of the growth factor (TGFB-1) plays a crucial role in mediating renal fibrosis. Signalling TGF-B1 antagonizing may be useful for the treatment of kidney disease [3].

Sirtuin-1 (SIRT-1) is a nicotine-amide adenine dinucleotide-dependent deacetylase. SIRT-1 is a crucial molecule in glucose, lipid, and energy metabolism. The renal protective effect of SIRT-1 is found in renal disorders with metabolic impairment, such as DN. Protective effects include the maintenance of glomerular barrier function, anti-fibrosis effects, anti-oxidative stress effects, and regulation of mitochondria function and energy

metabolism [10]. Oxidative stress is mainly due to the continuous production of free radicals, reactive oxidative stress (ROS), that imbalances with free radicals and antioxidant system production. It is negatively associated with cell viability, energy metabolism, aging, and metabolic and degenerative diseases. SIRT-1 is involved in several cellular functions, including chromosomal stability, cell cycle, apoptosis, DNA repair, metabolism, and aging by deacetylation of various transcription factors (NF-kB, P53, FOXO), histone and non-histone proteins [11].

SIRT-1 deficiency under stress conditions such as metabolic or oxidative stress is implicated in the pathophysiology of cardiovascular diseases, diabetes, neurodegenerative disorders, and renal disease. SIRT-1 may inhibit renal cell apoptosis, inflammation, and fibrosis in the kidneys. The activation of SIRT-1 in the kidney may be a new therapeutic target to increase resistance to many causal factors in developing renal diseases, including DN [12]. Since SIRT-1 is an essential metabolic sensor, its activity is regulated dynamically to allow for adaption and alteration to the cellular metabolic state. Nutritional, hormonal, and environmental signals, as well as the NAD+ level and SIRT-1 interacting proteins responding to those signals, compose the regulation network of SIRT-1. With a high-glucose and high-fat diet, SIRT-1 expression decreases, while during starvation and nutrient deprivation, SIRT-1 expression increases. During the stress response, SIRT-1 links chromatin dynamics/ gene expression to environmental stimuli [13]. SIRT-1 controls cellular transcription and metabolism, with a consequent crucial role in adaptation to oxidative, gen-toxic, or metabolic stresses [14].

Furthermore, scientific data indicates that the inflammatory factors tumor necrosis factor (TNF-α) and interleukin (IL)-6 are well reported to contribute to renal impairment in diabetes [15]. Probiotics appear to reduce inflammation and oxidative stress markers [16]. Additionally, Diabetes and obesity are both metabolism disorders associated with a low-grade inflammatory state. TNF-α may be a factor in the glomerular and interstitial tubule damage seen in diabetes [17]. According to a recent study, inhibiting TNF-α is a possible therapeutic method for experimental diabetic rats. These cytokines can be produced in diabetic kidneys by invading macrophage cells or by renal cells that are inherent to the kidney, like as endothelial cells, mesangial cells, glomerular cells, and tubular cells [18]. Therefore, antioxidative stress and anti-inflammation activity of some natural substances are essential approaches for preventing and treating renal fibrosis in DN.

Dadiah is considered a traditional food in the Minangkabau region, West Sumatra, Indonesia. Its benefits as a probiotic are supported by evidence regarding health and well-being. In addition, this *dadiah* is an important halal product for the Muslim population in the region. Therefore, biochemical and microbiological composition in *dadiah* is fundamental to learning to know the basic properties of health and disease prevention developments. *Dadiah Lintau* has been identified and has probiotic characteristics rich in lactic acid bacteria (LAB) with lactic acid bacterial composition 7.1 x 10^{10}. Based on molecular identification results using 16S rRNA methods and BLAST analysis, it has a similarity of 99.99% with *Lactobacillus fermentum* [19]. Another study has founded *L. plantarum* in *dadiah* sampled from Agam Bukittinggi West Sumatra [20].

Many studies are conducted by local and national researchers on the nutritional components and antimicrobial activity of dadiah. However, not many are clinically studied and scientifically proven their effects on various diseases. In addition, *dadiah* is also known to have characteristics of a probiotic with peptide components as antioxidants that can stimulate endogenous antioxidants in the host body [21]. Therefore, the use of antioxidants in the case of DM should be considered to prevent the development of DM into DN. Therefore, finding "probiotic products" to treat DN is necessary. This study aims to prove that *dadiah* has the potential as an activator of SIRT-1 to prevent the progressivity of DN through the repair of kidney tissue.

2. Materials and Methods

2.1. Research Design

This research consists of three continuing stages: *In vitro, in silico, and in vivo*. *In vivo* study is an experimental study base on animal trials with a post-test-only control-group design**.** This study has been approved by the Ethics Committee of Medical Faculty of Baiturrahmah University (No: 001/ETIK-FKUNBRAH/03/03/21).

2.1.1. Preparation of *Dadiah*

Dadiah's samples were taken from buffalo milk the village of Tanjung Bonai, Tanah Datar Regency, West Sumatra. Identification of specimen *dadiah* is carried out in the laboratory of animal husbandry biotechnology/Technology animal product. The *dadiah* was obtained from Lintau, West Sumatra. The dosage of administration, based on the recommended dosage of fermented milk in humans with a body weight of 70 kg, was 100-200 mL per day [22]. The density (ρ) of *dadiah* was 1.04 g/mL, with the formula:

Density = mass (g) / volume (mL)

Mass = 1.04 g/mL x 100 mL = 104 g of *dadiah*

Thus, the recommended *dadiah* dosage: 104 - 208 g/70 kg of human.

From the Laurence table (2008), the conversion value of 70 kg of human weight to 200 g of rat weight is 0.018, thus the calculation of *dadiah* dosage for rat (1), (2), (3):

Dadiah dosage for rat = conversion value x *dadiah* dosage for human (1)

= 0.018 x 104 = 1.87 g/200 g of rat weight

1.87 g of Dadiah/200 g of Rat weight = 9.35 g/kg b.w

Dadiah dosage (g/mL) for treatment 1: K $= \frac{9.35\ \text{g/kg b. w x } 0.2\ \text{Kg}}{\text{mL}} = 0.935\ \text{g/mL}$ (2)

The weight of male white rat (*Rattus norvegicus*): ± 300 g = 0.3 Kg

Administered volume (mL) = 9.35 g/kg b.w x 0.3 Kg = 3 ml/ day (3)

0.935 g/m

Dadiah solution containing 1 g/mL was made by suspending *dadiah* with *aquadest*.

2.1.2. Preparation of Lactic Acid Bacteria (LAB)

Isolate *L. fermentum* is rejuvenated first, then propagated in the medium Mann Rugose Sharpe (MRS) broth at a temperature of 37°C for 24 hours and calculated the number of bacterial cells by diluting up to 10^8 CFU / ml. Dilution results are calculated on the MRS medium so that it is incluted at a temperature of 37°C for 2x24 hours in the incubator, to find out the number of LAB to be induced.

2.1.3. Preparation of a Cell-Free Supernatant (Bacteriocin)

The LAB of *dadiah* were cultivated in MRS broth (1000 ml) seeded with 10% inoculum of overnight culture and incubated at 37°C for 24 hour. Following incubation, the entire broth was centrifuged for 16 minutes at 10,000 X g for 16 minutes and the cell-free supernatant was used as crude bacteriocin [23].

2.2. In Vitro study

This research was conducted as a preliminary study to prove that *dadiah* has characteristics of a probiotic. The results obtained are macroscopic identification, microscopic identification, biochemical tests, acid and bile salt resistance assays, antimicrobial tests and identification LAB with 16S rRNA [19].

2.2.1. Macroscopic Identification

Media dilution that is used is de MRS broth. Results of dilution BAL done with spread method, at inoculation and stored in anaerobic jar after its incubation in incubator for 48 hours at a temperature of 37°C. Single colony that characterize BAL is round, smooth white yellowish colour were then transferred to de Mann ROGOSA Sharpe MRS media for purification of colony by streak method and incubated for 24 hours at a temperature of 37°C [24].

2.2.2. Microscopic Identification

Bacterial culture was taken in a Petri dish using an inoculation needle, then put into a glass preparation. Added drops of crystal violet. Wait for one minute, then rinsed with distilled water and dried, then drops of iodine was added, and wait 1 minute, Rinse with distilled water and dried, then dipped in ethanol for ± 20 minutes. One drop of safranin is added. Wait 30 seconds, rinse and dry and observe the shape of bacteria under the microscope [25].

2.2.3. Biochemical Properties

By adding LAB isolates into 5 ml of MRS BRC MERCK (Merck), the gas test was performed. Then, invert the Durham tube and incubate at 37 °C for 48 hours, observing for the presence or absence of air bubbles in the Durham tube. Next, the catalase test is performed by scraping the isolation to the glass preparation and dropping 3 percent (v/v) hydrogen peroxide (H_2O_2) on a microscope slide for the bacterial review [26].

2.2.4. Acid Resistance Test

1 mL bacterial culture was added to 9 mL MRS Broth media and incubated at 37°C for 24 hours. Then, up to 1 mL of bacterial culture was added to a reaction tube containing 9 mL MRS Broth without pH control (control) or MRS Broth pH 3 (pH regulated with HCl 5N) and incubated for 90 minutes. Finally, pH three and control cultures were diluted to 10-6 and spread onto MRS media for 48 hours at 37°C. The colony forming unit (CFU) determined the maximum number of bacteria that can survive. Cell viability has been selected by comparing their numbers before and after incubation [21].

2.2.5. Bile Salt Resistance Test

1 ml of bacterial culture was added to 9 ml MRS Broth medium and incubated at 37°C for 4 hours with ox gal settings of 0.5 percent (w/v). The culture was then diluted to 10^{-6} and inoculated on MRS media using the

spread method. It was then incubated for 48 hours at 37°C. The number of bacteria capable of survival was determined using the cup count method with the CFU [27].

2.2.6. Antimicrobial Test

The disk diffusion method was used to determine antimicrobial activity against *Escherichia coli* O157, *Listeria monocytogenes*, and *Staphylococcus aureus* ATCC 25923 microorganisms. A 1 mL LAB culture was placed in sterile Eppendorf tubes and of the LAB supernatant [20].

2.2.7. Identification LAB by 16S rRNA [28].

2.3. In Silico Study

The in silico method is used in the bioinformatics test to determine the number of things; The probiotic *L. fermentum* produces other metabolites and substances; Discovering the pathways associated with the target protein being studied in a laboratory that influence the development of DN; Determining which protein interactions have the most impact on biological processes that occur in DN. The test were Analysis of pathways with KEGG on STRING, Prediction of Target Proteins with SEA, Interaction Proteins with DB STRING and Analysis of Metabolite compounds using WAY2Drug PASS server.

2.3.1. Prediction of Target Proteins with SEA

The similarity ensemble approach search tool (SEArch), one of the most widely used drug discovery tool based on the similarity of binding ligands for *in silico* target prediction, was used to predict target proteins for each compound from *Lactobacillus fermentum*'s metabolite and other compounds. The SMILE string of the listed compounds was inputted into SEArch (http://sea.bkslab.org/search/). The selected results of target proteins were associated to DN. The potential hit was evaluated by E-values and **Tanimoto coefficient** (Tc). The suggested threshold of E-values and Max Tc was 10^{-4} and 0.57. The result of E values greater than the limit was not considered into the study, as they did not indicate great statistical significance [29,30].

2.3.2. Interaction Proteins with DB STRING

STRING DB (https://string-db.org/) was used to predict protein-protein interaction. Protein-protein interaction was used to understand physiology and to determine their efficacy. Homo sapiens database and high confidence score 0.7 used as minimum required interaction score. False Discovery Rate usedto describes how significant the enrichment is. Shown are p-values corrected for multiple testing within each category using the Benjamín-Hochberg procedure. Smallest P-value are the most significant [31].

2.3.3. Analysis of Metabolite compounds using WAY2Drug PASS server

Secondary metabolites in LAB literature study results analyzed its potential using WAY2DRUG PASS prediction. (http://www. pharmaexpert. ru/ passonline/predict.php) as diabetic treatment. Previously, each compound needed to be searched smile structure (simplified molecular-input line-entry system) obtained from pub-chem database (https://pubchem.ncbi.nlm.nih.gov/). Then the compound analyzed its potential using WAY2DRUG PASS prediction to find out its potential in DN. The Pa (Probability to be Active) value describes the potential of the compound being test. Determination of value is comparing the structure of compounds with compounds that have proved as a specific treatment. Potential Analysis has done using Way2Drug Pass Server. The Pa value is 0.7 more indicates that the compound is predicted to have a high potential as anti-diabetic. The high similarity with the compounds in the database has been proven as such treatments. Whereas the value of Pa is more than 0.3 but less than 0.7, then the compound computationally has low similarity to the compound that has been proven as the treatment [32].

2.4. In Vivo Study

2.4.1. The Nurture of Experimental Animal

The Wistar-strain male white rats (*Rattus norvegicus*) were first adapted for 2 weeks before being treated. Rats were placed in a cage with husk mat to absorb dirt. The cage was placed in a sufficiently ventilated room at a temperature of 20-26°C. The cage was cleaned every day. 30-40 g of standard foods was given each day for each animal and the drink was added with *ad libitium.*

2.4.2. The Treatment Phase

Before the experiment, we measured all rats' blood glucose levels by cutting 1 mm off their tails. Blood was then dropped on a glucometer (OneTouch Merck; accuracy ISO 15197:2003) and UriScan Test Strips for proteinuria (Biosys Laboratories, INC). After collecting all the data, we performed the first experiment that caused hyperglycemia (>200 mg/dL) and proteinuria in rats. Experimental rats were made diabetic by injecting i.p (intraperitoneal) alloxan 100 mg/kg [33].

A preliminary study found that Alloxan's dosage could cause DN in rats eight days after injection. On the eighth day, mice were injected with Alloxan to check blood sugar and urine protein levels with UriScan. Trial

mice with blood glucose levels above 200 mg/dl were randomly grouped in this study. Six groups of diabetic rats were created. One group received only aquadest (Control Positive), while the other received *dadiah* 3 gr a day in aqua solution (P1) and LAB 1 ml and 2 ml once a day for P2 and P3 groups. In addition, P4 and P5 received 1 and 2 ml bacteriocin. Thus, on day 8 of treatment, P1-P5 mice will receive it. Control groups (C- and C+) were given water and food ad libitum. Eight weeks of *dadiah*, LAB, and Bacteriocin were administered. Dissection was performed after 8 weeks of treatment is given.

2.4.3. Animal Termination Phase

Male white rats (*Ratus norvegicus*) were sacrificed by means of Anasthesia with ether. The method was by mixing the concentrated ether solution with 2% NaCl solvent or 10-25% in NaCl and a dose of 300 mg/kg or 1-1.25 g/kg. Identification and nephrectomy were carried out, then directly put into a 10% BNF solution. After the kidney organ was removed, neck pressure was done to kill it while pulling it anteriorly (*dislocasio atlanto-occipitalis*) [34]. Examine dependent variables based on immunohistochemistry and histopathological profiles.

2.4.4. Laboratory Examination phase

2.4.4.1. Tissue Processing

Rat renal tissue was processed into paraffin blocks and cut with a microtome with a thickness of 4 mm. The preparations were stained with haematoxylin-eosin and sirius red. Measurements were taken by photo-shooting haematoxylin-eosin preparations with Olympus BX 51 light microscope at 400x (objective 40x) and 1000x (objective 100x) magnifications. Photomicrographs were taken in representative areas.

The paraffin block was cut with a 4µm-thick rotary microtome, then placed on a slide. Deparaffinized it with Xylene for 5 minutes, twice (2 x 5 minutes). Rehydrated it with graded alcohol, started by 100%, 96%, and 70% ethanol, then distilled water, 5 minutes for each. The preparation was stained with hematoxylin for 8 minutes. Rinsed it with aquadest for 10 minutes. Dehydrated it with 70% alcohol for 5 minutes. Then with 96% alcohol for 5 minutes. Next, immersed the preparation in Eosin Y solution for 2 minutes. Rinsed it in 96% ethanol for 5 minutes. then, with 100% Ethanol for 5 minutes. Cleared it in Xylene for 5 minutes, twice. Mounted the deck glass with entellant.

2.4.4.2. Hematoxillin-Eosin Procedure

The paraffin block was cut with a 4µm-thick rotary microtome, then placed on a slide. Deparaffinized it with Xylene for 5 minutes, twice (2 x 5 minutes). Rehydrated it with graded alcohol, started by 100%, 96%, and 70% ethanol, then distilled water, 5 minutes for each. The preparation was stained with hematoxylin for 8 minutes. Rinsed it with aquadest for 10 minutes. Dehydrated it with 70% alcohol for 5 minutes. Then with 96% alcohol for 5 minutes. Next, immersed the preparation in Eosin Y solution for 2 minutes. Rinsed it in 96% ethanol for 5 minutes. then, with 100% Ethanol for 5 minutes. Cleared it in Xylene for 5 minutes, twice. Mounted the deck glass with entellant. (ScyTek Laboratories Procedure)

2.4.4.3. Immunohistochemistry Procedure (IHC)

The paraffin block was cut with a rotary microtome with a 4µm-thick rotary microtome, then placed on a glass coated with Poly-L-lysine. Deparaffinized it with Xylene, then rehydrated it with graded alcohol, started by 100%, 96%, and 70% ethanol, then distilled water, 5 minutes for each. The heat induced retrieval epitope used a microwave for 10 minutes inside Citrate buffer at pH 6. Washed it in phosphate buffer saline (PBS) at pH 7.4 for 5 minutes, three times. The endogenous peroxidase blocking was with 3% H_20_2 in PBS at pH 7.4 for 3 minutes, followed by 0.3% H_20_2 in PBS at pH 7.4 for 30 minutes. Washed it in PBS at pH 7.4 for 5 minutes, three times. The non-specific protein block was with 2% NGS (Normal Goat serum) in PBS at pH 7.4 for 20 minutes, at room temperature. Applied the primary antibody and incubated it in a 4°C humid chamber over-night. TNF-α dilution 1; 100, and Anti SIRT-1 antibody (EPR 18239) ab 189494, ABCAM; dilution 1; 50. Washed it in PBS at pH 7.4 for 5 minutes, three times. Incubated it with secondary antibodies at room tempera-ture for 30 minutes. Washed it in PBS at pH 7.4 for 5 minutes, three times. Incubated it with the avidin biotin complex at room temperature for 30 minutes (ScyTek Laboratories Procedure)

2.4.4.4. Assessing Fibrocollagen Matrix Deposition in Sirius Red Staining

The collagen matrix was stained red on the Sirius red staining. The area measurement was done by taking a photomicrograph at 400x magnification (40x objective) in 5 different fields. The red-stained area was measured using the ImageJ program (ImageJ v1.49 software, National Institute of Health, Bethesda, MD, USA) by isolating the red-stained area on the Sirius red staining, and then calculating the colored area proportion to the field of view area; the positive-colored area was reported in percentage (Kiernan JA. Sirius Red Staining Protocol for Collagen. MedEmoryEdu).

2.4.4.5. Assessing the Expressions of and SIRT-1 and TNF on the IHC Staining

The expressions of SIRT-1 and TNF-α appeared brown on the IHC staining. The staining pattern was mainly in the form of cytoplasmic staining. The SIRT-1 and TNF-α expression was calculated cell positive in percentage with ImageJ based on quantitative assessment methods. It has been shown using the Olympus BX51 light microscope at 400x magnification (40x objective). The area has been evaluated for intracytoplasmic brown staining. Rats tissue was observed from five different fields of view. In each field of view, the staining intensity was reported in 4 levels (negative, weak, moderate, and strong) (ABCAM Procedure Antibody Kit SIRT-1 and TNF-α)

2.5. Data Analyze

Comparison The test was conducted using the average difference test, namely the one-way ANOVA test (for more than 2 treatment groups). Before the test, the underlying assumption was the normality of the data the Kolmogorov-Smirnov test. If the data used does not meet any or all the assumptions, a replacement test will be conducted, that is, the Kruskal Wallis test. If the results of the one-way ANOVA are significantly different, the Duncan test will be carried out, as well as the further test for the Kruskal Wallis test, that is, Mann Whitney. If the notation of the results of the further test between the two treatments is different, then the two treatments are significantly different. Meanwhile, if the notation between the two treatments is the same, then the two treatments are not significantly different test between treatments.

3. Results

The results of the normality test showed that each significance value of the variable fibril-collagen matrix deposition with Sirius red (interstitial fibrosis) was greater than 0.05, then a decision will be to accept H0, which means the data was normally distributed. The normally distributed data will be continued with the one-way ANOVA analysis. However, data for the variable Mn-SOD expression and SIRT-1 expression, were not normally distributed with each significance value of less than 0.05. Data that are not normally distributed were continued with Kruskal Wallis analysis.

The normally distributed data will be continued with the one-way ANOVA analysis. However, data for the variable SIRT-1 expression and TNF-α expression were not normally distributed with each significance value of less than 0.05. Data that are not normally distributed were continued with Kruskal Wallis analysis. The results of normality test can be seen in Table 1.

Table 1. The normality test

Variable	Statistic	Significance
TNF-α Expression	0.352	0.000
Sirtuin-1 Expression	0.169	0.004
Matrix deposition fibril-collagen with Sirius-red (Glomerular-sclerosis)	0.131	0.068*

3.1. In Vitro Study

This research was conducted as a preliminary study to prove that *dadiah* has characteristics of a probiotic. The results obtained are Macroscopic identification found Colony of LAB: white beige, round shape, size 1,8 mm, surface smooth and convex, total LAB count 7.1 x 10^{10} CFU/g. Gram staining revealed that LAB from *dadiah* contained rod-shaped and gram-positive bacteria. Biochemical test of *dadiah* were negative catalase, and homofermentative. Percentage acid resistance viability 57.1% and bile salt resistance viability 66.7%. *E. coli* possessed had the largest inhibition zone (23.28 mm), the inhibitory activity of *dadiah* LAB against *E. coli* is classified as very strong. The PCR results and BLAST analysis, the isolated bacteria from *dadiah* had 99.99% similarity with *L. fermentum* [19].

3.2. In Silico Study

The results study of *in vitro* above, identification of isolated LAB from *dadiah* using 16S rRNA, had 99.99% similarity with *L. fermentum*. Furthermore, the researcher conducted bioinformatics studies as the base on experimental test in the next stage. *L fermentum* is a species of lactic acid-producing bacteria and evidenced by many literature studies that show that these bacteria also have a variety of other metabolite compounds. *L fermentum* is a species of lactic acid-producing bacteria and evidenced by many literature studies that show that these bacteria also have a variety of other metabolite compounds such as; Glutathione (Keiser et al., 2007), Riboflavin (Thakur & Tomar, 2016), Vitamin K2 (menaquinone) by Lim et al., 2011, and according Hati et al., 2019 it has several compounds such as acetic acid, B9, B12 and butyric acid. The others study showed L fermentum also containing ferulic acid (Westfall & Lomis, 2016), Propionic acid,

Caproic acid, valerate, iso-butyrate, iso-and valerate (Pereira et al., 2003). Exopolysaccharide also finding in L fermentum (Santiago-López et al., 2018), and several compounds such as ethyl-pentadecanoate, linoleic acid and vaccenic acid (Yoon et al., 2020) and Matsuguchi et al., 2003.

3.2.1. Pathway DN Based on KEGG

Pathway analysis on diabetes complications with target proteins related to DN found three major pathways, namely AGE-RAGE signalling, FOXO signalling, and longevity regulating pathway.

3.2.2. Protein-Ligan Network Analysis

Based on the protein-protein interaction (PPI) approach, search target proteins are involved in the mechanism of diabetes nephropathy AGE-RAGE signalling pathway (NFKB1, TGF, TNF), FOXO signalling pathway (EP300, SOD, SIRT), and longevity signalling pathway (NFKB1, SIRT, SOD).

The resulting potential protein-ligand network in this study showed ferulic acid, caproic acid, linoleic acid, and vaccenic acid suggested metabolite compound in *L. fermentum* were selected results of target proteins associated with DN pathways. The potential hit was evaluated by E-values and Tanimoto coefficient (Tc). The suggested threshold of E-values and Max-Tc was 10^{-4} and 0.57, respectively [35]. The result of E-values greater than the limit was not considered into the study, as they did not indicate great statistical significance [36].

In Fig. 1, several target proteins have a high score of PPI String, which is related to the DN pathway of the metabolite compound *L. fermentum*. Target proteins directly related to DN pathways are described as being in outer circles such as NF-κB, JUN, EP 300, PPARA, F3, MMP2, MMP9, NFE2L2, TLR2, TLR4 and PPARG. While based on the highest score of protein interaction (PPI Score), proteins closely related to the target protein that can be studied through laboratory studies are TNF-α and SIRT-1 (the inner circle). This protein computationally has high confidence if conducted in vivo and in vitro testing with results that have affected the occurrence of diabetes complications (DN) through the pathways set in KEGG. Below is described the biological activity of target proteins in DN with a significant p-value rate (Benjamin-Hochberg). Some studies showed that SIRT-1 has a reno-protective impact on ND through deacetylation of transcription factors involved in renal disease pathogenesis. Recently, it has been found that specific overexpression of SIRT-1 by podocyte cells may decrease proteinuria and kidney injury in experimental mice with ND [37]. SIRT-1 is involved in several cellular functions, including chromosomal stability, cell cycle, apoptosis, DNA repair, metabolism, and aging by deacetylation of various transcription factors (NF-kB, P53, FOXO) histone and non-histone proteins [11]. This target protein is associated with metabolite compounds through its various biological activities, shown by the color shown in the picture and table above.

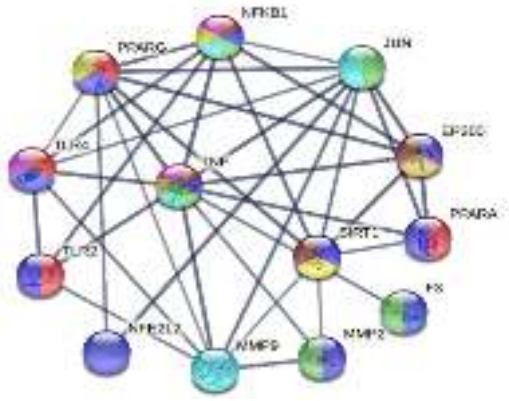

Fig. 1 High score PPI

In Table 2, the target protein is seen with pathways that play a role in DN. The lowest yield (p-value 0.00000000292) is the most significant seen in the path of "Regulation of response to stress" with the target protein, namely, NFKB1, EP300, PPARA, F3, MMP2, NFE2L2, TLR2, TLR4, PPARG, TNF, and SIRT-1 (blue coloring). Each target protein can have some biological activity, as seen in Fig. 2.

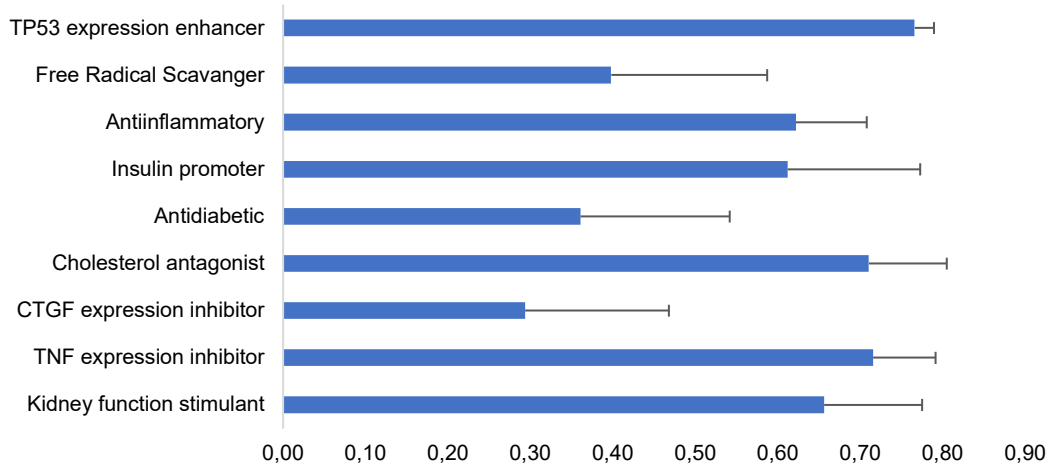

Fig. 2 Biological process of metabolites compound

Table 2. Role of Target Proteins in DN pathways by secondary compounds in *L. fermentum* (PPI STRING)

Pathway	False discovery rate Benjamini-Hochberg (p-value)	Color	Protein
Regulation of inflammatory response	0.00000017		**NFKB1 PPARA TLR2 TLR4 PPARG TNF**
Regulation of response to stress	0.00000000292		**NFKB1 EP300 PPARA F3 MMP2 NFE2L2 TLR2 TLR4 PPARG TNF SIRT1**
AGE-RAGE signaling pathway in diabetic complications	0.0000000061		**NFKB1 JUN F3 MMP2 TNF**
NF-kappa B signaling pathway	0.000230		**NFKB1 TLR4 TNF**
TGF signaling pathway	0.000180		**EP300 PPARG NFKB1**
TNF signaling pathway	0.0000103		**MMP9 TNF NFKB1 JUN**
FOXO signaling pathway	0.00047		**SIRT1 EP300 TNF**
Longevity signaling pathway	0.0002000		**SIRT1 PPARG NFKB1**

Secondary metabolites in *L. fermentum* literature study results analyzed its potential using WAY2DRUG PASS prediction. (http://www.pharmaexpert.ru/passonline/ predict.php) as diabetic treatment. Previously, each compound needed to be searched smile structure (simplified molecular-input line-entry system) obtained from pub-chem database (https://pubchem.ncbi.nlm.nih.gov/). Then the compound analyzed its potential using WAY2DRUG PASS prediction to find out its potential in DN. The Pa (probability to be active) value describes the potential of the compound being test. Determination of value is comparing the structure of compounds with compounds that have proved as a specific treatment. Potential Analysis has done using Way2Drug Pass Server. The Pa value is 0.7 more indicates that the compound is predicted to have a high potential as anti-diabetic. The high similarity with the compounds in the database has been proven as such treatments. Whereas the value of Pa is more than 0.3 but less than 0.7, then the compound computationally has low similarity to the compound that has been proven as the treatment.

3.3. In Vivo Study

3.3.1. The Expression of SIRT-1 by Immunohistochemistry in Kidneys of Experimental Animals

The expressions of SIRT-1 appeared brown on the IHC staining. The staining patterns was mainly in the form of cytoplasmic staining. The staining assessment used the semiquantitative assessment technique, using the IRS criteria. The microscopic assessment used the Olympus BX51 light microscope at 400x magnification (40x objective) by assessing the positive intracytoplasmic brown staining on the representative area. Each sample was observed in 5 different fields of view. In each field of view (40x objective), the proportion of epithelial cells with positive intracytoplasmic brown staining was calculated, then compared to all epithelial cells per field of view; the staining intensity was reported in 4 intensity levels (negative, weak, moderate, and strong). SIRT-1 immunohistochemical staining of experimental animal kidney tissue; negative control group (a, h), positive control (b, i), treatment with curd (c, j), low-dose LAB (d, k), high-dose (e, l), and low-dose bacteriocin (f, m) and high-dose

(b, i) (Fig. 3). SIRT-1 was stained brown, mainly with the matrix staining pattern around the glomerulus and tubules. There was a decrease in the SIRT-1 expression in the alloxan induction group. The treatment of *dadiah*, lactic acid bacteria, and bacteriocin showed a higher expression of SIRT-1 than the positive control. Immunoperoxidase, low magnification with 10x objective lens (top), and high magnification with 40x objective lens (bottom) 200µm scale. The number of SIRT-1 expression in each treatment can be seen in Table 3 and Fig. 4.

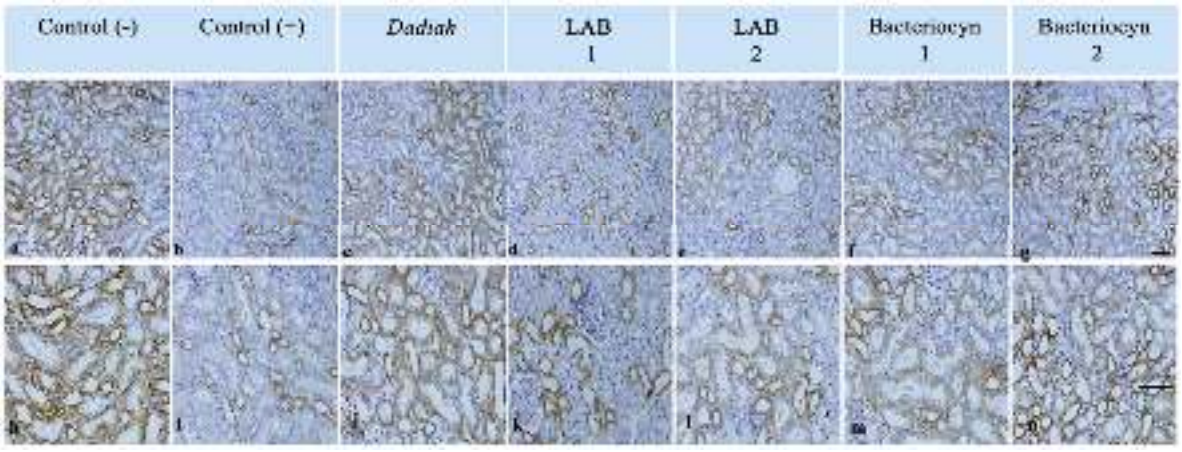

Fig. 3 The assessment of SIRT-1 expression by immunohistochemistry

Table 3. The average number of SIRT-1 expression in each treatment (% positive cells)

Samples	Average	Standard Deviation	Notation
Negative Control (C-)	80.0000	0.00000	d
Positive Control (C+)	36.6667	5.16398	a
P1	83.3333	5.16398	d
P2	51.6667	7.52773	b
P3	61.6667	20.41241	bc
P4	63.3333	19.66384	bc
P5	66.6667	12.11060	c
Chi-square count	= 26.131		
p-value	= 0.000		

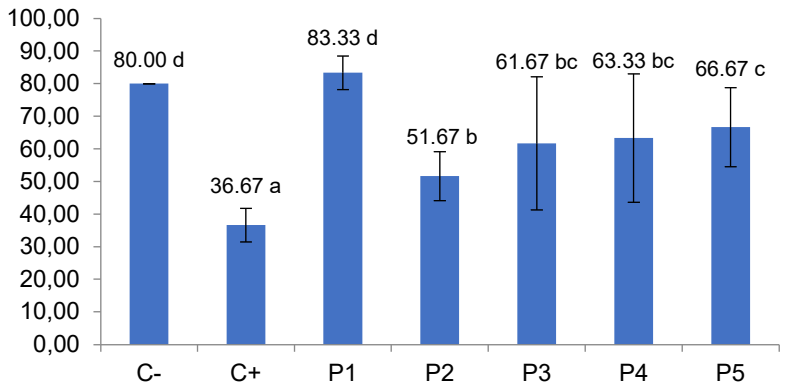

Fig. 4 SIRT-1 expression numbers in each treatment

Most studies have established the crucial effects of SIRT-1 deacetylase in protecting kidney cells from stress. SIRT-1 has been shown to protect podocytes and kidney tubular cells in a variety of kidney illness situations, including DN. Sirt-1 protects against DN in part by deacetylating disease-associated transcription factors such as p53, FOXO, p65, NF-kB, and STAT3. Recently, it was demonstrated that induction of SIRT-1 in podocytes significantly improved proteinuria and renal damage in an experimental DN model [37].

Due to the critical role of SIRT-1 as a metabolic sensor, its activity is dynamically regulated to allow for alteration to changes in the cellular metabolic state. SIRT-1's regulation network is comprised of nutritional,

hormonal, and environmental cues, as well as the NAD+ level and SIRT-1 interacting proteins that respond to these signals.

SIRT-1 expression is decreased in response to a high-glucose, high-fat diet, but it is raise in response to famine and food deprivation [38–40]. SIRT-1 establishes a connection between chromatin dynamics/gene expression and environmental cues during the stress response. SIRT-1 activation may assist the kidney in metabolic conditions such as diabetes mellitus. Wakino et al. [10] demonstrated that reduced SIRT-1 in the proximal tubules represents the initiation of DN using animal models of diabetes mellitus. Additionally, SIRT-1 is implicated in the pathogenesis of DN [13]. SIRT-1 a NAD+-dependent protein deacetylase, participates in various physiological activities, including hypoxia stress, DNA repair, cell aging, inflammatory, and mitochondria regulation; yet, its degradation is required for the formation of ND. SIRT-1 expression was significantly decreased in the renal of diabetic db/db rats in previous research. Recent research, however, indicates that SIRT-1 is involved in the endoplasmic reticulum stress response to hyperglycemia and hypoxia [41].

3.3.2. The Expression of TNF-α by Immunohistochemistry in Kidneys Tissues

The expressions of TNF-α, appeared brown on the IHC staining. The staining pattern was mainly in the form of cytoplasmic staining. The proportion of epithelial cells with positive intracytoplasmic brown staining was calculated, then compared to all epithelial cells per field of view; the staining intensity was reported in 4 intensity levels (negative, weak, moderate, and strong).

The staining of TNF-α immunohistochemistry in the kidney tissue of animal model; the negative control group (a, h) and the positive control (b, i), the treatment with *dadiah* (c, j), the low-dosage LAB (d, k) and the high dosage (e, l), and the low-dosage bacteriocin (f, m) and the high dose (b, i) (Fig. 5). The TNF-α was stained brown in some tubular epithelial cells and some cells in the stroma, with a weak staining in the matrix around the glomeruli and tubules. There was an increase in the TNF-α expression in the alloxan induction group, both in epithelial and stromal cells. The administration of *dadiah*, lactic acid bacteria, and bacteriocin, showed lower TNF- α expression than the positive control. The average number of TNF expression in each treatment can be seen in Table 4.

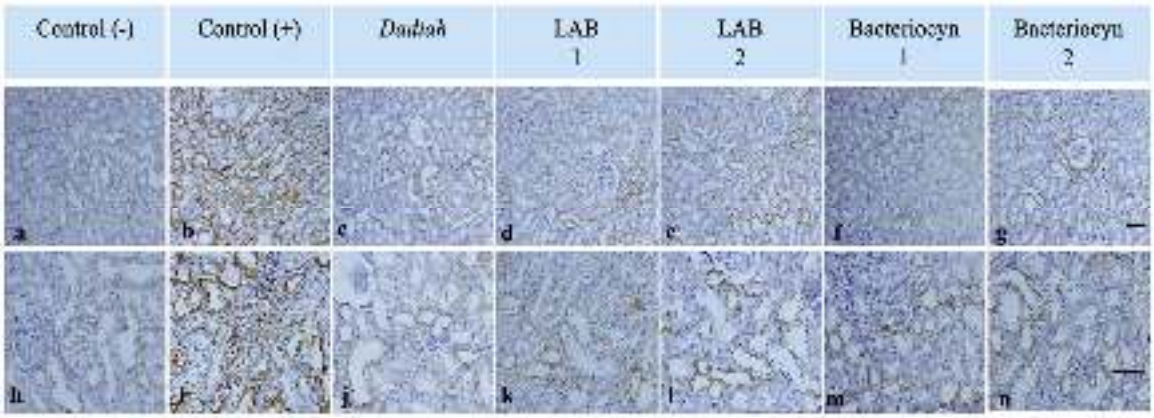

Fig. 5 The assessment of TNF-α expression with immunohistochemistry

Based on Fig. 6, it can be seen that the highest average of TNF expression in the C+ group (induced by alloxan + proteinuria) was 76.67±5.16, and the lowest average of TNF expression was in the C – group (not induced by alloxan and not given any treatment), which was equal to 16.67±5.16. To prove whether there was a statistically significant difference in the average number of TNF expression, the Kruskal Wallis statistical analysis would be carried out.

Table 4. The average number of TNF expression in each treatment

Sample	Average	Standard Deviation	Notation
C–	16.6667	5.16398	a
C+	76.6667	5.16398	d
P1	20.0000	6.32456	ab
P2	23.3333	12.11060	abc
P3	30.0000	0.00000	c
P4	26.6667	8.16497	bc
P5	23.3333	10.32796	abc
Chi-square count	= 24.362		

p-value	= 0.000

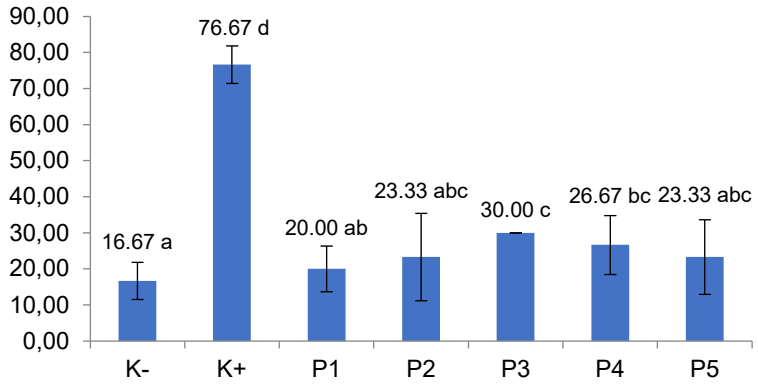

Fig. 6 TNF-α expression numbers in each treatment

Based on the results of the Kruskal Wallis test, the p-value was smaller than (0.000 < 0.050), so it can be concluded that there is a significant difference in the average TNF expression number between treatments. To see the difference, further tests were carried out using the Mann Whitney test with the results notation in Table 5. It can be seen that:

The highest average of TNF expression in the C+ treatment was significantly different from the C-, P1, P2, P3, P4, and P5 treatments. The lowest average of TNF expression in C- groups was significantly different from C+, P3, and P4 treatment groups, but C- groups was not significantly different from P1, P2, and P5 treatment groups.

Table 5. Average of fibrosis interstitial fibrosis in the groups

Samples	Average	Standard Deviation	Notation
Negative Control (C-)	12.0667	0.78145	a
Positive Control (C+)	17.6667	0.90480	c
P1	14.9333	1.50687	b
P2	15.2833	1.95900	b
P3	15.8167	1.98133	b
P4	15.1833	1.79490	b
P5	15.0667	1.18434	b
F count	= 7.117		
p-value	= 0.000		

Additionally, scientific data indicates that the inflammatory factors TNF-α and IL-6 are well reported to contribute to renal impairment in diabetes [15]. Probiotics appear to reduce inflammation and oxidative stress markers, according to a growing body of studies [16]. Diabetes and obesity are both metabolism disorders associated with a low-grade inflammatory state. TNF-α is a marker inflammation cytokine that has been shown to phosphorylate the insulin receptor's serine residue substrate (IRS-1), inactivating it, while IL-1, TNF-α, and interferon (IFN) are known to function synergistically by invading the pancreas and generating-cell damage and apoptosis [42–44]. In STZ-induced diabetic rats, *Lactobacillus casei* strain Shirota significantly reduced pro-inflammatory cytokines IL-6, IL-4, and CRP. In a diabetes kidney rat model fed for 12 weeks, a probiotic mixture decreased TNF-α and increased IL-10. Similar studies have demonstrated the anti-inflammatory benefits of probiotic lactobacilli. Thus, our findings corroborate previous reports indicating that *L. fermentum* spp. had anti-inflammatory properties [45].

3.3.3. The Deposition of Fibro-Collagen Matrix with HE Sirius Red (Interstitial Fibrosis)

The connective tissue staining of the experimental animal kidneys with sirius red stained showed the interstitial and periglomerular connective tissue. The connective tissue matrix was stained with magenta. Negative control group (a, h), positive control (b, i), treatment with *dadiah* (c, q), low-dose LAB (d, k), high-dose (e, l), low-dose bacteriocin (f, m) and high-dose (b, i) (Fig. 7). The collagen deposition was measured using the ImageG program by extracting the red area, converting the image to black and white, and measuring the percentage area of the coloured area per unit area. The collagen deposition was lower in the experimental animals with *dadiah* treatment, lactic acid bacteria, and bacteriocin treatment, compared with the positive controls. The lowest collagen deposition was in the *dadiah* treatment, compared to other treatments.

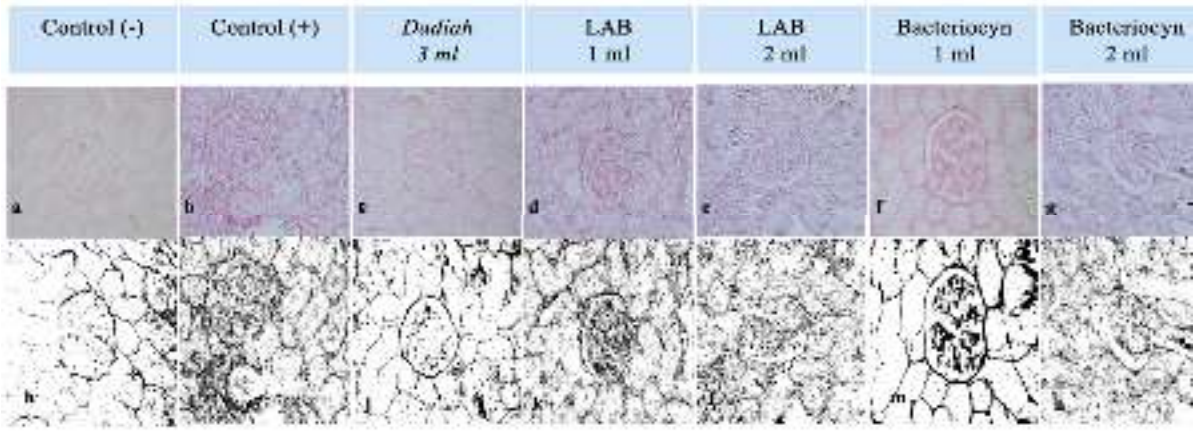

Fig. 7 Deposition of fibro-collagen matrix in kidneys with Sirius Red staining

Induction with alloxan administration showed an increase in collagen matrix deposition in the renal parenchyma as a sign of glomerulosclerosis [17,46].

Based on Fig. 8, it can be seen that the highest average of glomerular fibrosis rate in the C+ treatment (induced by alloxan + proteinuria) was 17.67 ± 0.90, and the lowest average of glomerular fibrosis was in the C– treatment (not induced by alloxan and not given a treatment), namely of 12.07 ± 0.78. The one-way ANOVA statistical analysis would be used to determine how a statistically significant difference in the average number of glomerular fibrosis existed. The one-way ANOVA test resulted in a p-value less than (0.000< 0.050), indicating a statistically significant difference in the average number of interstitial fibrosis between treatments. To demonstrate the distinction, more tests were conducted using the Duncan test and the notation results in Table 5. It can be seen that: The highest average of glomerular fibrosis in the C+ treatment was significantly different from the C-, P1, P2, P3, P4, and P5 treatments. The lowest average of kidney fibrosis in the C- treatment was significantly different from the C+, P1, P2, P3, P4, and P5 treatments.

The activation of metabolic, inflammatory, and hemodynamic pathways describes the pathophysiology of DKD. For example, chronic hyperglycaemia leads to increased PKC activity, alterations in polyol metabolism, increased secretion of profibrotic cytokines (such as TGF-B1), and non-enzymatic glycosylation glycation of glomerulosclerosis structures and the formation of AGEs. The accumulation of aberrant protein glycation markers is a significant factor in the onset and progression of DKD. AGEs increase in the mesangial and glomerular capillary walls in people with DN, according to immunohistochemistry findings. The kidney plays a crucial role in AGEs metabolism [9]. Renal fibrosis, myofibroblast, podocyte dysfunction, basement membrane thickening, and extracellular matrix protein build-up are all regarded to be standard features of DN. Podocytes are a type of high differentiation glomerular epithelial cell that has been linked to the early pathogenic mechanism of DN pathogenesis [47–51]. Furthermore, the increase in inflammation directly destroys renal function [49]. In diabetes, the deposition of advanced glycation end products (AGEs) plays a crucial role in the development of DN. Additionally, inflammation and peroxidation are associated with the onset and progression of DN, respectively. The main characteristics of DN include a thick basement membrane, mesangial expansion, podocyte loss in the glomerular, and increased urine microalbumin excretion. Further, ECM protein build-up plays a vital role in developing DN. DN is also characterized by renal fibrosis and glomerular sclerosis [3].

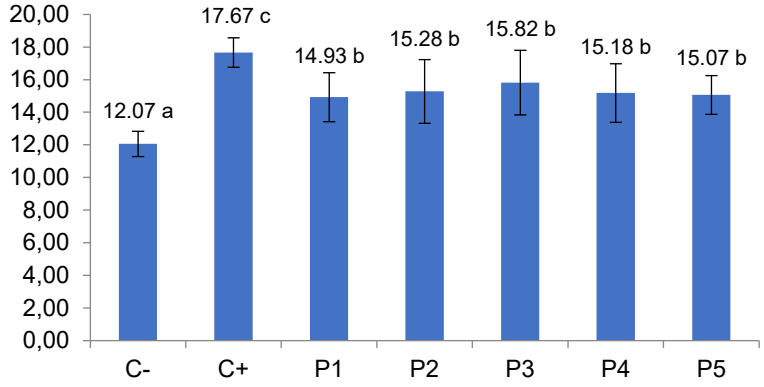

Fig. 8 The proportion of fibrocolagen matrix in the kidney tissue

4. Discussion

4.1. In Vitro Study

LAB in *dadiah* was 7.1 10^{10}. In comparison to probiotics from Prato cheese, which have a vibrant color [51]. According to Emmawati [52], the LAB isolate from *Mandai* is a fermented product made of *cempedak* (*Artocarpus champeden*) dami. *Mandai* samples has the total number of LAB as probiotic food. The other study, found 14 isolates LAB has the total colony result are the dilution of 10^{-7} is 1,25 x 10^{9} CFU/g and the dilution of 10^{-8} is 3,0 x 10^{8} CFU/g. The identification with macroscopic in MRS agar medium is seen the sign of colony is circle of the whole, broken white, round shape, convex, edge slick and small and big size [53]. Other study from fresh goat milk samples located in Western and North Western provinces of Sri Lanka, found the most of the isolated colonies were creamy circular in shape with wet surface, raised with entire margins [24]. Additionally, this study compared LAB research on kefir created from the fermentation of fresh milk with the addition of kefir grains as carrier components for probiotic organisms to an indigenous LAB source. The bacteria in kefir have a population density of between 6.4x10^{4} and 8,5x10^{8} CFU/g [54]. Microscopic identification showed The presence of LAB and its efficacy as probiotic sources in a traditional fermented foods was proved using *Cyprinus carpio*, *Dengke Naniura* of *Bataknese*, Indonesia. The identification of LAB morphology was found Gram-positive, bacilli, cocci, and bacilli cocci [56,57]. According to the catalase assay, the LAB isolate used in biochemical test does not produce catalase. The study reported no presence of bubbles and stated that the LAB from *dadiah* from Lintau Buo is homofermentative. The observation results are not seen there is a gel reservoir on the LAB, it is demonstrated that the catalase assay tool is significantly negative. According from Ibrahim [58] reported to the findings, LAB isolated from mango exhibited a negative catalase assay result.

Thus, acid resistance assays on *dadiah* LAB at pH 4 and 3 were performed. The control samples had a more significant number of colonies to grew (7 × 10^{8} CFU/L than the pH 3 (4 × 10^{8} CFU/ml), with a survival rate of 57.1%. The viability value changes according to the type of bacteria that can live at low pH and the strain of bacteria. Along with acid resistance, probiotics require LAB resistance to bile salts. The 0.5 percent concentration is sufficient to select for bile salt-resistant strains [59]. The LAB from dadiah demonstrated a significant antimicrobial effect of harmful microbiota. The results indicated that LAB from *dadiah* possessed an inhibitory effect on *E. coli* to kanamycin and ampicillin. According to Morales [60], the zone of inhibition is classified as weak (less than 5 mm), medium (5-10 mm), strong (>10-20 mm), and very strong (>20-30 mm). Thus, the inhibitory activity of *dadiah* LAB against *E. coli* is classified as very strong.

According to the PCR and BLAST analyses, the isolated bacteria from *dadiah* were 99.99 percent identical to *L. fermentum*.

Like other studies, Meekiri back-slopping, a traditional Sri Lankan food obtained from fermented buffalo milk products, also has several strains such as *L. fermentum, L. curvatus, and L. acidophilus, and L. plantarum*. In Sri Lanka, milk fermentation gel is obtained using a back-sloping technique that is a simple technique using a small inoculum derived from the previous coagulum as a culture starter in the selection of BAL strains [26].

Research about different isolations carried out by Syukur and Fachrial [61] obtained the *L. plantarum* bacteria isolated from *dadiah* from *Sijunjung*, in which the base length was 1525 bp. Similarly, according to the studies undertaken by Purwati et al. [53] the isolation and characterization of LAB from *dadiah* also resulted in *L. plantarum strain Dad-13*, which had a similarity value of 97–100 percent when BLAST analysis was used [54]. The research of Melia and Purwati [62] on buffalo milk samples from the *Agam* district (BMA 3.3) reported the classification LAB using BLAST analysis *as a strain of L. fermentum (L23)*. Sequencing results showed that 41.6 percent (5 isolates) were identified as *Lactococcus lactis* ssp. *lactis*, 25 percent (3 isolates) identified as *Lactobacillus plantarum* ssp. *plantarum*, 16.6 percent (2 isolates) identified as *L. lactis* ssp. *cremoris*, and 8.3 percent (1 isolate each) identified as *Pediococcus pentosaceus* and *Lactobacillus pentosus* [63]. This study is in line with research conducted by Sukma [64], wherein the LAB in *dadiah* was dominated by bacteria from the *Lactococcus*, *Lactobacillus*, and *Leuconostoc* groups.

4.2. In Silico Study

Pathway analysis on diabetes complications with target proteins related to DN found three major pathways, namely AGE-RAGE Signalling, FOXO Signalling, and Longevity Regulating Pathway, with results as seen in links: (AGE-RAGE Signalling pathway in diabetic complications); (FOXO Signalling pathway); (Longevity regulating pathway).

4.2.1. Protein-Ligan Network Analysis

The resulting potential protein-ligand network in this study showed ferulic acid, caproic acid, linoleic acid, and vaccenic acid suggested metabolite compound in *L. fermentum* were selected results of target proteins associated with DN pathways (Table 2). The potential hit was evaluated by E-values and Tanimoto coefficient (Tc). The suggested threshold of E-values and Max-Tc was 10^{-4} and 0.57, respectively [35]. The result of E- values greater than the limit was not considered into the study, as they did not indicate great statistical significance [36].

4.2.2. Protein-Protein Interaction by STRING DB

Target proteins directly related to DN pathways are described as being in outer circles such as NF-□B, JUN, EP 300, PPARA, F3, MMP2, MMP9, NFE2L2, TLR2, TLR4 and PPARG. While based on the highest score of protein interaction (PPI Score), proteins closely related to the target protein that can be studied through laboratory studies are TNF and SIRT-1 (the inner circle). This protein computationally has high confidence if conducted in vivo and in vitro testing with results that have affected the occurrence of diabetes complications (DN) through the pathways set in KEGG. Below is described the biological activity of target proteins in DN with a significant p-value rate (Benjamin-Hochberg). Some studies showed that SIRT-1 has a reno-protective impact on DN through deacetylation of transcription factors involved in renal disease pathogenesis. Recently, it has been found that specific overexpression of SIRT-1 by podocyte cells may decrease proteinuria and kidney injury in experimental mice with ND [36]. SIRT-1 is involved in several cellular functions, including chromosomal stability, cell cycle, apoptosis, DNA repair, metabolism, and aging by deacetylation of various transcription factors (NF-kB, P53, FOXO) histone and non-histone proteins [11]. The target protein is seen with pathways that play a role in DN. The lowest yield (p-value 0.00000000292) is the most significant seen in the path of "Regulation of response to stress" with the target protein, namely, NFKB1, EP300, PPARA, F3, MMP2, NFE2L2, TLR2, TLR4, PPARG, TNF, and SIRT-1 (Table 3).

4.2.3. Bioactive and Metabolites Compounds Potential *L. fermentum* as DN Treatment

Secondary metabolites in *L. fermentum* literature study results analyzed its potential using WAY2DRUG PASS prediction. (http://www.pharmaexpert.ru/ passonline/ predict.php) as diabetic treatment. Previously, each compound needed to be searched smile structure (simplified molecular-input line-entry system) obtained from pub-chem database (https://pubchem.ncbi.nlm.nih.gov/). Then the compound analyzed its potential using WAY2DRUG PASS prediction to find out its potential in DN. The Pa (Probability to be Active) value describes the potential of the compound being test. Determination of value is comparing the structure of compounds with compounds that have proved as a specific treatment. Potential analysis has done using Way2Drug Pass Server. The Pa value is 0.7 more indicates that the compound is predicted to have a high potential as anti-diabetic. The high similarity with the compounds in the database has been proven as such treatments. Whereas the value of Pa is more than 0.3 but less than 0.7, then the compound computationally has low similarity to the compound that has been proven as the treatment.

Fig. 2 have seen the potential of metabolite compound *L. fermentum* in the incidence of DN with a significant score of>0.7 will have high potential, while the score 0.5-0.7 has a moderate potential effect on DN computationally.

Suppose the average score of various metabolite compounds produced by *L. fermentum* in literature studies with biological processes occurs. In that case, the metabolite compounds of *L. fermentum* with a computational influence are lactic acid compounds with a score of 0.579 and ferulic acid compounds 0.580. While the most instrumental biological activity is TP53 expression enhancer (0.77) and TNF-α expression inhibitor (0.72), this is following several in vivo studies that state that inflammatory processes are an essential mechanism of dm progressivity into DN, so that by inhibiting TNF expression and increased expression of TP53, it can inhibit inflammatory processes in diabetes, so that microvascular complications will be inhibited. The study showed that high circulating TNF receptor levels might be e a new indicator of DN. TNF-α receptors 1 and 2 are critical, independent predictors for the production of macroalbuminuria in DN [9]. Inflammatory cytokines such as IL-1, IL-6, IL-18, TNF-α have been linked to the development and progression of DN [65].

4.3. In Vivo Study

SIRT-1 a NAD+-dependent protein deacetylase, participates in various physiological activities, including hypoxia stress, DNA repair, cell aging, inflammatory, and mitochondria regulation; yet, its degradation is required for the formation of ND. SIRT-1 expression was significantly decreased in the renal of diabetic db/db rats in previous research. Recent research, however, indicates that SIRT-1 is involved in the endoplasmic reticulum stress response to hyperglycemia and hypoxia [41]. Most studies have established the crucial effects of SIRT-1 deacetylase in protecting kidney cells from stress. SIRT-1 has been shown to protect podocytes and kidney tubular cells in a variety of kidney illness situations, including DN. Sirt-1 protects against DN in part by deacetylating disease-associated transcription factors such as p53, FOXO, p65, NF-kB, and STAT3. Recently, it was demonstrated that induction of SIRT-1 in podocytes significantly improved proteinuria and renal damage in an experimental DN model [37].

Additionally, scientific data indicates that the inflammatory factors tumor necrosis factor TNF-α and IL-6 are well reported to contribute to renal impairment in diabetes [41]. Probiotics appear to reduce inflammation and oxidative stress markers, according to a growing body of studies [16]. In STZ-induced diabetic rats, Lactobacillus casei strain Shirota significantly reduced pro-inflammatory cytokines IL-6, IL-4, and CRP. In a diabetes kidney rat model fed for 12 weeks, a probiotic mixture decreased TNF-α and increased IL-10.

Similar studies have demonstrated the anti-inflammatory benefits of probiotic lactobacilli. Thus, our findings corroborate previous reports indicating that *L. fermentum* spp. had anti-inflammatory properties [45].

The activation of metabolic, inflammatory, and hemodynamic pathways describes the pathophysiology of DN. For example, chronic hyperglycaemia leads to increased PKC activity, alterations in polyol metabolism, increased secretion of profibrotic cytokines (such as TGF-B1), and non-enzymatic glycosylation glycation of glomerulosclerosis structures and the formation of AGEs. The accumulation of aberrant protein glycation markers is a significant factor in the onset and progression of DKD. AGEs increase in the mesangial and glomerular capillary walls in people with DN, according to immunohistochemistry findings. The kidney plays a crucial role in AGEs metabolism [9]. Renal fibrosis, myofibroblast, podocyte dysfunction, basement membrane thickening, and ECM protein build-up are all regarded to be standard features of DN.

Furthermore, the increase in inflammation directly destroys renal function [5]. In diabetes, the deposition of AGEs plays a crucial role in the development of DN. Additionally, inflammation and peroxidation are associated with the onset and progression of DN, respectively. The main characteristics of DN include a thick basement membrane, mesangial expansion, podocyte loss in the glomerular, and increased urine microalbumin excretion. Further, ECM protein build-up plays a vital role in developing DN. DN is also characterized by renal fibrosis and glomerular sclerosis [3].

5. Conclusions

Oral administration of *dadiah* and probiotics and secondary metabolite compounds of LAB have been shown to increase the production of SIRT-1 and reducing the TNF-α expression that marker in stress oxidative and inflammatory processes caused by hyperglycaemia, and therefore alleviating renal fibrosis.

Administration of *dadiah* solution, isolate probiotic strain *L. fermentum*, and isolate *bacteriocin* from *dadiah* has been shown to ameliorate renal tissue fibrosis in DN mice when stained with Sirius-red. In addition, oral administration of *dadiah*, probiotics and secondary metabolite compounds of lactic acid bacteria showed to increase the expression of SIRT-1 and reduced TNF-α, which functions were to reduce stress oxidative and inflammatory processes caused by hyperglycaemia, and therefore alleviating renal fibrosis. The findings of this study could be to develop novel treatments for DN that aim to reduce the cascade of oxidative stress and inflammatory signals in kidney tissue.

5.1. Study Limitation

- Proteinuria examined in this study was measured qualitatively using UriScan. Urine measurements should be quantitative by Radioimmunoassay (RIA) to be statistically analyzed for their effect on the administration of dadiah and its metabolites.
- Bacteriocin isolated from probiotics *dadiah* is not pure bacteriocin but contains other metabolite components produced by lactic acid bacteria *dadiah* (free supernatant cell).
- In the experimental stage study, researchers only looked at the relationship between variable oxidative stress and inflammation to changes in the anatomical pathology structure of kidney tissue with DN. DN is a complex event partially mediated and modified by genetic factors, lifestyle, and environmental exposure (epigenetic).
- No examination of other metabolite compounds contained in *dadiah* with *Spectrophotometer* method.

5.2. Future Study

This research is still being done on experimental animals, so it is necessary to conduct further research for clinical trials in humans. Clinical trials are essential in proving the effect of *dadiah* on kidney function improvement in DN patients who are known to have damage and death of glomerular podocyte cells that cause proteinuria in DM. The examination of this clinical trial can be done using urine samples. The various examinations include macroalbuminuria and microalbuminuria, the number of podocyte cells in the urine (podosituria), angiotensinogen, and nephrin. In addition, serum creatinine examination can also do to show the glomerular filtration rate (e-LFG). This study only examined the potency of *dadiah* and its metabolites against inflammatory repair parameters and antioxidant effects on DN without comparing it to antidiabetic drugs. Further study experimental research with comparing to anti-diabetic drugs.

Statements and Declarations

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Disclosure of potential conflicts of interest: The authors have no competing interests to declare that are relevant to the content of this article.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of Medical Faculty of Baiturrahmah University (No: 001/ETIK-FKUNBRAH/03/03/21).

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The Anti-inflammatory potential of probiotic *Dadiah* to activate Sirtuin-1 in inhibiting Diabetic Nephropathy progression

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Abstract

Background: Diabetic Nephropathy (DN) is a serious microvascular complication of DM. The activation of SIRT-1 in the kidney has become a new therapeutic target to increase resistance to many causal factors in DN development. Furthermore, antioxidative stress and anti-inflammation are essential to preventing renal fibrosis in DN. Therefore, finding "probiotic products" to treat and prevent DN is necessary.
Aim: This study proved the Anti-inflammatory Potential of Probiotic Dadiah to Activate SIRT-1 in Inhibiting DN Progression.
Material and Methods: This study is an experimental group designed with a post-test-only control group to observe the effect of dadiah, LAB, and bacteriocin on alloxan-induced nephropathy diabetic rats.
Results: The treatment of dadiah, lactic acid bacteria, and bacteriocin showed a higher expression of Sirtuin-1 than the positive control. The SIRT-1 expression was higher in the dadiah treatment compared to other treatments. They also, reduce TNF-α expression varies significantly between treatments. The highest average of interstitial fibrosis in the C+ groups was substantially different from all groups, but all treatments showed decreased kidney fibrosis. Although all treatments showed a decrease in interstitial kidney fibrosis found in the control group, the treatment using dadiah showed the highest result.
Conclusion: *Dadiah* has the potential to the prevention of fibrosis on kidney tissue of alloxan-induced nephropathy diabetic rats. The findings could be to develop novel treatments for DN that aim to reduce the cascade of oxidative stress and inflammatory signals in kidney tissue

Keywords: *Dadiah,* Sirtuin-1, TNF-α, Diabetic Nephropathy

1. Introduction

Diabetes Mellitus (DM) is one of the most significant health problems worldwide. According to the projections, the number of adult diabetic patients will exceed 430 million in 2030. Diabetic Nephropathy is one of the most microvascular complications and is now the leading cause of end-stage renal disease (ESRD)(Chen et al., 2020; Guilbaud et al., 2020; Kundu et al., 2020; Zheng et al., 2020). The prevalence of DM is increasing and is an essential cause of microvascular diseases such as Diabetic nephropathy (Y. J. Li et al., 2020). Diabetic Nephropathy (DN) is a serious microvascular complication of DM, and according to data in the United States, it is estimated to be suffered by 44% (30 - 40%) DM patients (Kundu et al., 2020). The main criteria to diagnose DN is the presence of an increased urinary albumin excretion (UAE), which is divided into microalbuminuria and macroalbuminuria, which is associated with an increased risk of decline in glomerular filtration rate (GFR) and a high risk of kidney failure(Pérez-Morales et al., 2018).Natural-history studies show the occurrence of proteinuria, eventually develops in 30-50% of diabetic persons (Nagarajrao & Alharbi, 2018); (Seaquist et al., 2010). Many pathways involving DN, such as hyperglycaemia, oxidative stress (OS), and protein kinase C (PKC) activation, have been postulated. As a significant mediator for DN development and progression, the upregulation of AGE receptors (RAGE) (Macisaac et al., 2014). Renal fibrosis, characterized by extracellular matrix protein accumulation, leads to CKD, including DN. It found that the process of signalling transformation of the growth factor (TGFB-1) plays a crucial role in mediating renal fibrosis. Signalling TGF-B1 antagonizing may be useful for the treatment of kidney disease (Kundu et al., 2020).

Figure 1. The Progression of Nephropathy Diabetes (Kundu *et al.*, 2020)

Dadiah is considered a traditional food in the Minangkabau region, West Sumatra. Its benefits as a probiotic are supported by evidence regarding health and well-being. In addition, this dadiah is an important halal product for the Muslim population in the region. Therefore, biochemical and microbiological composition in dadiah is fundamental to learning to know the basic properties of health and disease prevention developments. Dadiah Lintau has been identified and has probiotic characteristics rich in lactic acid bacteria with lactic acid bacterial composition 7.1 x 10^{10}. Based on molecular identification results using 16S rRNA methods and BLAST analysis, it has a similarity of 99.99% with *Lactobacillus fermentum* (Amelia et al., 2021).

Many studies are conducted by local and national researchers on the nutritional components and their antimicrobial activity. However, not many are clinically studied and scientifically proven their effects on various diseases. In addition, dadiah is also known to have characteristics of a probiotic with peptide components as antioxidants that can stimulate endogenous antioxidants in the host body (Harun et al., 2020). Therefore, the use of antioxidants in the case of DM should be considered to prevent the development of DM into diabetes nephropathy.

Sirtuin-1 is a nicotine-amide adenine dinucleotide-dependent deacetylase. Sirtuin-1 is a crucial molecule in glucose, lipid, and energy metabolism. The renal protective effect of Sirtuin-1 is found in renal disorders with metabolic impairment, such as diabetic nephropathy. Protective effects include the maintenance of glomerular barrier function, anti-fibrosis effects, anti-oxidative stress effects, and regulation of mitochondria function and energy metabolism (Wakino et al., 2015). Oxidative stress is mainly due to the continuous production of free radicals (ROS) that imbalances with free radicals and antioxidant system production. It is negatively associated with cell viability, energy metabolism, aging, and metabolic and degenerative diseases. Sirtuin-1 is involved in several cellular functions, including chromosomal stability, cell cycle, apoptosis, DNA repair, metabolism, and aging by deacetylation of various transcription factors (NF-kB, P53, FOXO), histone and non-histone proteins (Balaiya, 2017). SIRT-1 deficiency under stress conditions such as metabolic or oxidative stress is implicated in the pathophysiology of cardiovascular diseases, diabetes, neurodegenerative disorders, and renal disease. SIRT-1 may inhibit renal cell apoptosis, inflammation, and fibrosis in the kidneys. The activation of SIRT-1 in the kidney may be a new therapeutic target to increase resistance to many causal factors in developing renal diseases, including diabetic nephropathy (Kitada et al., 2013). Since SIRT-1 is an essential metabolic

sensor, its activity is regulated dynamically to allow for adaption and alteration to the cellular metabolic state. Nutritional, hormonal, and environmental signals, as well as the NAD+ level and SIRT-1 interacting proteins responding to those signals, compose the regulation network of SIRT-1. With a high-glucose and high-fat diet, SIRT-1 expression decreases, while during starvation and nutrient deprivation, SIRT-1 expression increases. During the stress response, SIRT-1 links chromatin dynamics/ gene expression to environmental stimuli (Guan & Hao, 2015). Sirtuin-1 controls cellular transcription and metabolism, with a consequent crucial role in adaptation to oxidative, gen-toxic, or metabolic stresses (Cencioni et al., 2020).

Additionally, scientific data indicates that the inflammatory factors tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) are well reported to contribute to renal impairment in diabetes (Song et al., 2020). Probiotics appear to reduce inflammation and oxidative stress markers (Mahmoodpoor et al., 2017).

Diabetes and obesity are both metabolism disorders associated with a low-grade inflammatory state. TNF-α may be a factor in the glomerular and interstitial tubule damage seen in diabetes (Navarro & Mora-ferna, 2006). According to a recent study, inhibiting TNF-α is a possible therapeutic method for experimental diabetic rats. These cytokines can be produced in diabetic kidneys by invading macrophage cells or by renal cells that are inherent to the kidney, like as endothelial cells, mesangial cells, glomerular cells, and tubular cells (Prigent *et al.*, 2000). Therefore, antioxidative stress and anti-inflammation are essential approaches for preventing and treating renal fibrosis in DN. Therefore, finding “probiotic products” to treat DN is necessary. This study aims to prove that dadiah has the potential as an activator of SIRT-1 to prevent the progressivity of DN through the repair of kidney tissue.

2. Materials and Methods

A. Material

Dadiah's samples were taken from buffalo milk the village of Tanjung Bonai, Tanah Datar Regency, West Sumatra. Identification of specimen dadiah is carried out in the laboratory of animal husbandry biotechnology/Technology animal product.

Preparation of Dadiah

The *dadiah* was obtained from Lintau, West Sumatra. The dosage of administration, based on the recommended dosage of fermented milk in humans with a body weight of

70 kg, was 100-200 mL per day (Harnavi Harun et al., 2020). The density (ρ) of dadiah was 1.04 g/mL, with the formula:

Density = mass (g) / volume (mL)

Mass = 1.04 g/mL x 100 mL = 104 g of dadiah

Thus, the recommended dadiah dosage: 104 - 208 g/70 kg of human.

From the Laurence table (2008), the conversion value of 70 kg of Human weight to 200 g of Rat weight is 0.018, thus the calculation of dadiah dosage for rat:

Dadiah dosage for rat = conversion value x dadiah dosage for human

= 0.018 x 104 = 1.87 g/200 g of rat weight

1.87 g of Dadiah/200 g of Rat weight = 9.35 g/kg b.w

Dadiah dosage (g/mL) for treatment 1: $K = \frac{9.35 \text{ g/kg b.w x } 0.2 \text{ Kg}}{2 \text{ mL}} = 0.935 \text{ g/mL}$

The weight of male white rat (*Rattus norvegicus*): ± 300 g = 0.3 Kg

Administered volume (mL) = $\frac{9.35 \text{ g/kg b.w x } 0.3 \text{ Kg}}{0.935 \text{ g/m}}$ =3 ml/ day

Dadiah solution containing 1 g/mL was made by suspending dadiah with *aquadest*.

Preparation of LAB

Isolate *L. fermentum* is rejuvenated first, then propagated in the medium MRS broth at a temperature of 37^0 C for 24 hours and calculated the number of bacterial cells by diluting up to 10^8 CFU / ml. Dilution results are calculated on the MRS medium so that it is incluted at a temperature of 37^0 C for 2x24 hours in the incubator, to find out the number of LAB to be induced

Preparation of a cell-free supernatant (Bacteriocin)

The LAB of dadiah were cultivated in MRS broth (1000 ml) seeded with 10% inoculum of overnight culture and incubated at 37° C for 24 hour. Following incubation, the entire broth was centrifuged for 16 minutes at 10,000 X g for 16 minutes and the cell-free supernatant was used as crude bacteriocin (Pato et al., 2021)

B. Methods

This research consists of three continuing stages: *In Vitro, In Silico, and In Vivo*. In vivo study is an experimental study base on animal trials with a post-test-only control-group design**.**

In Vitro study

This research was conducted as a preliminary study to prove that dadiah has characteristics of a probiotic. The results obtained are Macroscopic identification, Microscopic identification, Biochemical tests, Acid and bile salt resistance assays, antimicrobial tests and Identification Lactic Acid Bacteria with 16S rRNA.

In Silico study

The in silico method is used in the bioinformatics test to determine the number of things; The probiotic L fermentum produces other metabolites and substances; Discovering the pathways associated with the target protein being studied in a laboratory that influence the development of DN; Determining which protein interactions have the most impact on biological processes that occur in DN. The test were Analysis of pathways with KEGG on STRING, Prediction of Target Proteins with SEA, Interaction Proteins with DB STRING and Analysis of Metabolite compounds using WAY2Drug PASS server

In Vivo Study

Male Wistar rats (Rattus norvegicus) were adapted for two weeks before treatment. Rats were kept in a ventilated 20-26 ^{0}C cage. Rats ate 30-40 g of food daily and drank ad libitum. Before the experiment, we measured all rats' blood glucose levels by cutting 1 mm off their tails. Blood was then dropped on a glucometer (OneTouch Merck; accuracy ISO 15197:2003) and UriScan Test Strips for proteinuria (Biosys Laboratories, INC). After collecting all the data, we performed the first experiment that caused hyperglycemia (>200 mg/dL) and proteinuria in rats. Experimental rats were made diabetic by injecting i.p (intraperitoneal) alloxan 100 mg/kg (Szkudelski, 2001). A preliminary study found that Alloxan's dosage could cause diabetic nephropathy in rats eight days after injection. On the eighth day, mice were injected with Alloxan to check blood sugar and urine protein levels with UriScan. Trial mice with blood glucose levels above 200 mg/dl were randomly grouped in this study. Six groups of diabetic rats were created. One group received only aquadest (Control Positive), while the other received dadiah 3 gr a day in aqua solution (P1) and Lactic Acid Bacteria 1 ml and 2 ml once a day for P2 and P3 groups. In addition, P4 and P5 received 1 and 2 ml bacteriocin. Thus, on day 8 of treatment, P1-P5 mice will receive it. Control groups (C- and C+) were given water and food ad libitum. Eight weeks of dadiah, LAB, and Bacteriocin were administered. Dissection was performed after 8 weeks of treatment is

given. Male white rats (*Ratus norvegicus*) were killed by means of Anasthesia with ether. The method was by mixing the concentrated ether solution with 2% NaCl solvent or 10-25% in NaCl and a dose of 300 mg/kg or 1-1.25 g/kg. Identification and nephrectomy were carried out, then directly put into a 10% BNF solution. After the kidney organ was removed, neck pressure was done to kill it while pulling it anteriorly (*dislocasio atlanto-occipitalis*) (Rudy Agung Nugroho, 2018). Examine dependent variables based on immunohistochemistry and histopathological profiles.

Tissue Procesing

Rat renal tissue was processed into paraffin blocks and cut with a microtome with a thickness of 4 mm. The preparations were stained with haematoxylin-eosin and sirius red. Measurements were taken by photo-shooting haematoxylin-eosin preparations with Olympus BX 51 light microscope at 400x (objective 40x) and 1000x (objective 100x) magnifications. Photomicrographs were taken in representative areas.

The paraffin block was cut with a 4µm-thick rotary microtome, then placed on a slide. Deparaffinized it with Xylene for 5 minutes, twice (2 x 5 minutes). Rehydrated it with graded alcohol, started by 100%, 96%, and 70% ethanol, then distilled water, 5 minutes for each. The preparation was stained with hematoxylin for 8 minutes. Rinsed it with aquadest for 10 minutes. Dehydrated it with 70% alcohol for 5 minutes. Then with 96% alcohol for 5 minutes. Next, immersed the preparation in Eosin Y solution for 2 minutes. Rinsed it in 96% ethanol for 5 minutes. then, with 100% Ethanol for 5 minutes. Cleared it in Xylene for 5 minutes, twice. Mounted the deck glass with entellant.

Hematoxillin-Eosin Procedure

The paraffin block was cut with a 4µm-thick rotary microtome, then placed on a slide. Deparaffinized it with Xylene for 5 minutes, twice (2 x 5 minutes). Rehydrated it with graded alcohol, started by 100%, 96%, and 70% ethanol, then distilled water, 5 minutes for each. The preparation was stained with hematoxylin for 8 minutes. Rinsed it with aquadest for 10 minutes. Dehydrated it with 70% alcohol for 5 minutes. Then with 96% alcohol for 5 minutes. Next, immersed the preparation in Eosin Y solution for 2 minutes. Rinsed it in 96% ethanol for 5 minutes. then, with 100% Ethanol for 5 minutes. Cleared it in Xylene for 5 minutes, twice. Mounted the deck glass with entellant

Immunohistochemistry Procedure (IHC)

The paraffin block was cut with a rotary microtome with a 4µm-thick rotary microtome, then placed on a glass coated with Poly-L-lysine. Deparaffinized it with Xylene, then rehydrated it with graded alcohol, started by 100%, 96%, and 70% ethanol, then

distilled water, 5 minutes for each. The heat induced retrieval epitope used a microwave for 10 minutes inside Citrate buffer at pH 6. Washed it in PBS (phosphate buffer saline) at pH 7.4 for 5 minutes, three times. The endogenous peroxidase blocking was with 3% H_20_2 in PBS at pH 7.4 for 3 minutes, followed by 0.3% H_20_2 in PBS at pH 7.4 for 30 minutes. Washed it in PBS at pH 7.4 for 5 minutes, three times. The non-specific protein block was with 2% NGS (Normal Goat serum) in PBS at pH 7.4 for 20 minutes, at room temperature. Applied the primary antibody and incubated it in a 4^{o}C humid chamber overnight. TNF-α dilution 1; 100, and Anti SIRT1 antibody (EPR 18239) ab 189494, ABCAM; dilution 1; 50. Washed it in PBS at pH 7.4 for 5 minutes, three times. Incubated it with secondary antibodies at room temperature for 30 minutes. Washed it in PBS at pH 7.4 for 5 minutes, three times. Incubated it with the avidin biotin complex at room temperature for 30 minutes

Procedure for assessing fibrocollagen matrix deposition in sirius red staining

The collagen matrix was stained red on the Sirius red staining. The area measurement was done by taking a photomicrograph at 400x magnification (40x objective) in 5 different fields. The red-stained area was measured using the ImageJ program (ImageJ v1.49 software, National Institute of Health, Bethesda, MD, USA) by isolating the red-stained area on the Sirius red staining, and then calculating the colored area proportion to the field of view area; the positive-colored area was reported in percentage

Assessing the expressions of and SIRT-1 and TNF on the IHC staining

The expressions of SIRT-1 and TNF-α appeared brown on the IHC staining. The staining pattern was mainly in the form of cytoplasmic staining. The staining assessment used the semiquantitative assessment technique, using the IRS criteria

The microscopic assessment used the Olympus BX51 light microscope at 400x magnification (40x objective) by assessing the positive intracytoplasmic brown staining on the representative area.

Each sample was observed in 5 different fields of view. In each field of view (40x objective), the proportion of epithelial cells with positive intracytoplasmic brown staining was calculated, then compared to all epithelial cells per field of view; the staining intensity was reported in 4 intensity levels (negative, weak, moderate, and strong).

Table 1. Immunoreactive Score

Data Analyze

Comparison The test was conducted using the average difference test, namely the one-way ANOVA test (for more than 2 treatment groups). Before the test, the underlying assumption was the normality of the data the Kolmogorov-Smirnov test. If the data used does not meet any or all the assumptions, a replacement test will be conducted, that is, the Kruskal Wallis test. If the results of the one-way ANOVA are significantly different, the Duncan test will be carried out, as well as the further test for the Kruskal Wallis test, that is, Mann Whitney. If the notation of the results of the further test between the two treatments is different, then the two treatments are significantly different. Meanwhile, if the notation between the two treatments is the same, then the two treatments are not significantly different test between treatments

Research Ethics

Ethics clearance was approved by the Ethics Committee of Medical Faculty of Baiturrahmah University (No: 001/ETIK-FKUNBRAH/03/03/21

Results and Discussion

The results of the normality test showed that each significance value of the variable fibril-collagen matrix deposition with Sirius red (interstitial fibrosis) was greater than 0.05, then a decision will be to accept H0, which means the data was normally distributed. The normally distributed data will be continued with the one-way ANOVA analysis. However, data for the variable Mn-SOD expression and Sirtuin-1 expression, were not normally distributed with each significance value of less than 0.05. Data that are not normally distributed were continued with Kruskal Wallis analysis.

Table 2. The Normality Test

Variable	Statistic	Significance
TNF-α Expression	0.352	0.000
Sirtuin-1 Expression	0.169	0.004

Matrix deposition fibril-collagen with Sirius-red (Glomerular-sclerosis)	0.131	0.068*

The normally distributed data will be continued with the one-way ANOVA analysis. However, data for the variable SIRT-1 expression and TNF-α expression were not normally distributed with each significance value of less than 0.05. Data that are not normally distributed were continued with Kruskal Wallis analysis.

In Vitro study

This research was conducted as a preliminary study to prove that dadiah has characteristics of a probiotic. The results obtained are Macroscopic identification found Colony of LAB: white beige, round shape, size 1,8 mm, surface smooth and convex, total LAB count 7.1 x 10^{10} CFU/g. Gram staining revealed that LAB from *dadiah* contained rod-shaped and gram-positive bacteria. Biochemical test of *dadiah* were negative catalase, and homofermentative. Percentage acid resistance viability 57.1% and bile salt resistance viability 66.7%. E. coli possessed had the largest inhibition zone (23.28 mm), the inhibitory activity of dadiah LAB against E. coli is classified as very strong. The PCR results and BLAST analysis, the isolated bacteria from *dadiah* had 99.99% similarity with *L. fermentum* (Amelia et al., 2021).

In Silico study

The results study of *In Vitro* above, identification of isolated Lactic Acid Bacteria from dadiah using 16S rRNA, had 99.99% similarity with *L. fermentum.* Furthermore, the researcher conducted bioinformatics studies as the base on experimental test in the next stage. *L fermentum* is a species of lactic acid-producing bacteria and evidenced by many literature studies that show that these bacteria also have a variety of other metabolite compounds. Pathway analysis on diabetes complications with target proteins related to diabetic nephropathy found three major pathways, namely AGE-RAGE Signalling, FOXO Signalling, and Longevity Regulating Pathway. Based on the PPI (Protein-Protein Interaction) approach, search target proteins are involved in the mechanism of diabetes nephropathy AGE-RAGE signalling pathway (NFKB1, TGF, TNF), FOXO signalling pathway (EP300, SOD, SIRT), and Longevity signalling pathway (NFKB1, SIRT, SOD).

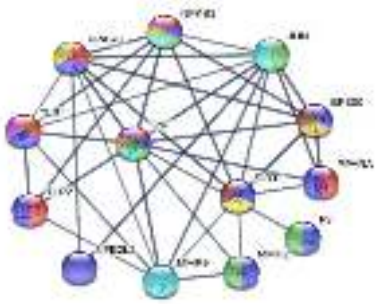

Figure 1 High score PPI

In Figure 1, several target proteins have a high score of PPI String, which is related to the Diabetic Nephropathy pathway of the metabolite compound L fermentum. Target proteins directly related to diabetic Nephropathy pathways are described as being in outer circles such as NF-κB, JUN, EP 300, PPARA, F3, MMP2, MMP9, NFE2L2, TLR2, TLR4 and PPARG. While based on the highest score of protein interaction (PPI Score), proteins closely related to the target protein that can be studied through laboratory studies are TNF and SIRT-1 (the inner circle). This protein computationally has high confidence if conducted in vivo and in vitro testing with results that have affected the occurrence of diabetes complications (diabetic nephropathy) through the pathways set in KEGG. Below is described the biological activity of target proteins in diabetic nephropathy with a significant p-value rate (Benjamin-Hochberg). Some studies showed that SIRT-1 has a reno-protective impact on ND through deacetylation of transcription factors involved in renal disease pathogenesis. Recently, it has been found that specific overexpression of sirt1 by podocyte cells may decrease proteinuria and kidney injury in experimental mice with ND (Zhong et al., 2018). Sirtuin-1 is involved in several cellular functions, including chromosomal stability, cell cycle, apoptosis, DNA repair, metabolism, and aging by deacetylation of various transcription factors (NF-kB, P53, FOXO) histone and non-histone proteins (Balaiya, 2017). This target protein is associated with metabolite compounds through its various biological activities, shown by the color shown in the picture and table above.

Table 3. Role of Target Proteins in diabetic Nephropathy pathways by secondary compounds in L. fermentum (PPI STRING)

Pathway	False discovery rate Benjamini-Hochberg (p-value)	Color	Protein
Regulation of inflammatory response	0.00000017		NFKB1 PPARA TLR2 TLR4 PPARG TNF
Regulation of response to stress	0.00000000292		NFKB1 EP300 PPARA F3 MMP2 NFE2L2 TLR2 TLR4 PPARG TNF SIRT1
AGE-RAGE signaling pathway in diabetic complications	0.0000000061		NFKB1 JUN F3 MMP2 TNF
NF kappa B signaling pathway	0.000230		NFKB1 TLR4 TNF
TGF signaling pathway	0.000180		EP300 PPARG NFKB1
TNF signaling pathway	0.0000103		MMP9 TNF NFKB1 JUN
FOXO signaling pathway	0.00017		SIRT1 EP300 TNF
Longevity signaling pathway	0.0002000		SIRT1 PPARG NFKB1

In table 3, the target protein is seen with pathways that play a role in diabetic nephropathy. The lowest yield (p-value 0.00000000292) is the most significant seen in the path of "Regulation of response to stress" with the target protein, namely, NFKB1, EP300, PPARA, F3, MMP2, NFE2L2, TLR2, TLR4, PPARG, TNF, and SIRT1 (blue coloring). Each target protein can have some biological activity, as seen in figure 2.

Secondary metabolites in *L. fermentum* literature study results analyzed its potential using WAY2DRUG PASS prediction. (http://www.pharmaexpert.ru/passonline/predict.php) as diabetic treatment. Previously, each compound needed to be searched smile structure (simplified molecular-input line-entry system) obtained from pub-chem database (https://pubchem.ncbi.nlm.nih.gov/). Then the compound analyzed its potential using WAY2DRUG PASS prediction to find out its potential in diabetic nephropathy. The Pa (Probability to be Active) value describes the potential of the compound being test. Determination of value is comparing the structure of compounds with compounds that have proved as a specific treatment. Potential Analysis has done using Way2Drug Pass Server. The Pa value is 0.7 more indicates that the compound is predicted to have a high potential as anti-diabetic. The high similarity with the compounds in the database has been proven as such treatments. Whereas the value of Pa is more than 0.3 but less than 0.7, then the compound computationally has low similarity to the compound that has been proven as the treatment.

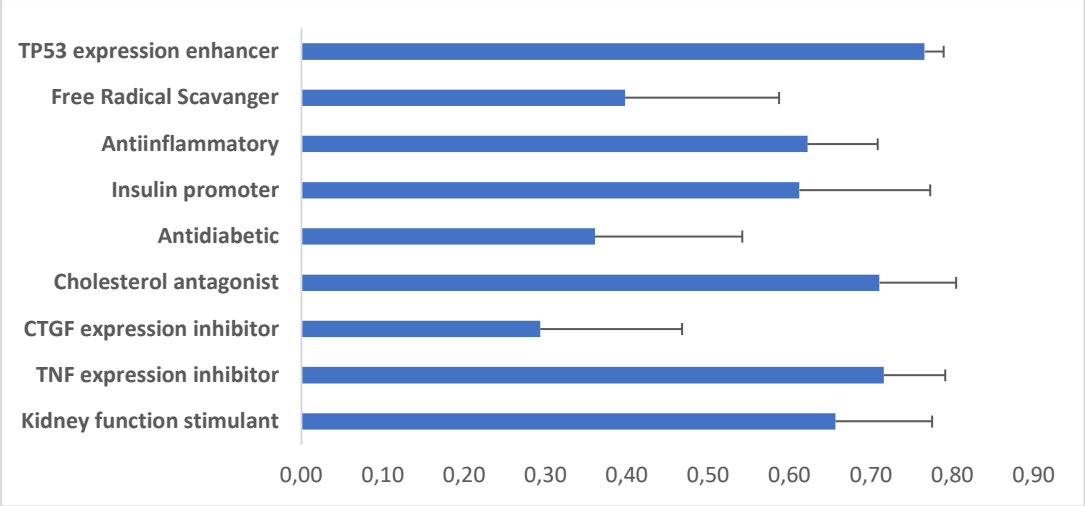

Figure 2. Biological process of metabolites compound

In Vivo Study

A. The Expression of SIRT-1 by Immunohistochemistry in Kidneys of Experimental Animals

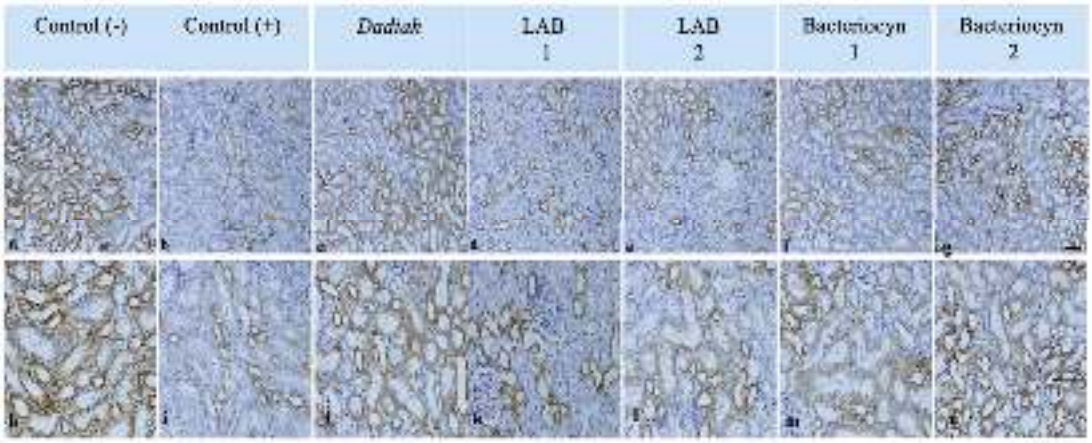

Figure 3. The Assessment of SIRT-1 expression by Immunohistochemistry

The expressions of SIRT-1 appeared brown on the IHC staining. The staining patterns was mainly in the form of cytoplasmic staining. The staining assessment used the semiquantitative assessment technique, using the IRS criteria. The microscopic assessment used the Olympus BX51 light microscope at 400x magnification (40x objective) by assessing the positive intracytoplasmic brown staining on the representative area. Each sample was observed in 5 different fields of view. In each field of view (40x objective), the proportion of epithelial cells with positive intracytoplasmic brown staining was calculated, then compared to all epithelial cells

per field of view; the staining intensity was reported in 4 intensity levels (negative, weak, moderate, and strong). Sirtuin-1 immunohistochemical staining of experimental animal kidney tissue; negative control group (a, h), positive control (b, i), treatment with curd (c, j), low-dose lactic acid bacteria (d, k), high-dose (e, l), and low-dose bacteriocin (f, m) and high-dose (b, i). Sirtuin-1 was stained brown, mainly with the matrix staining pattern around the glomerulus and tubules. There was a decrease in the Sirtuin-1 expression in the alloxan induction group. The treatment of *Dadiah*, lactic acid bacteria, and bacteriocin showed a higher expression of Sirtuin-1 than the positive control. Immuno-peroxidase, low magnification with 10x objective lens (top), and high magnification with 40x objective lens (bottom) 200µm scale.

Table 4 The average number of SIRT-1 expression in each treatment (% positive cells)

Samples	Average	Standard Deviation	Notation
Negative Control (C-)	80.0000	0.00000	d
Positive Control (C+)	36.6667	5.16398	a
P1	83.3333	5.16398	d
P2	51.6667	7.52773	b
P3	61.6667	20.41241	bc
P4	63.3333	19.66384	bc
P5	66.6667	12.11060	c
Chi-square count	= 26.131		
p-value	= 0.000		

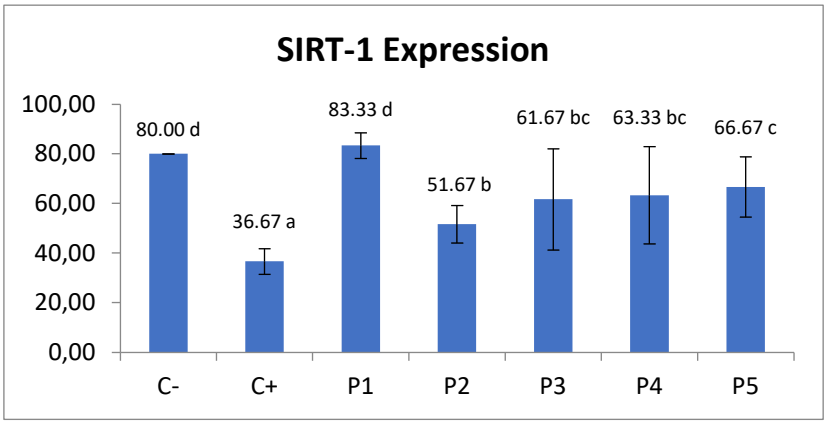

Figure 4 The graph of SIRT-1 expression numbers in each treatment

Most studies have established the crucial effects of Sirtuin-1 (SIRT1) deacetylase in protecting kidney cells from stress. SIRT1 has been shown to protect podocytes and kidney tubular cells in a variety of kidney illness situations, including diabetic

nephropathy (ND). Sirt-1 protects against ND in part by deacetylating disease-associated transcription factors such as p53, FOXO, p65, NF-kB, and STAT3. Recently, it was demonstrated that induction of SIRT1 in podocytes significantly improved proteinuria and renal damage in an experimental ND model (Zhong et al., 2018)

Due to the critical role of SIRT-1 as a metabolic sensor, its activity is dynamically regulated to allow for alteration to changes in the cellular metabolic state. SIRT-1's regulation network is comprised of nutritional, hormonal, and environmental cues, as well as the NAD+ level and SIRT-1 interacting proteins that respond to these signals. SIRT-1 expression is decreased in response to a high-glucose, high-fat diet, but it is raise in response to famine and food deprivation (Kane & Sinclair, 2018; Kong et al., 2015; Liu et al., 2017; Rabi Yacoub, Kyung Lee, 2017). SIRT-1 establishes a connection between chromatin dynamics/gene expression and environmental cues during the stress response. SIRT1 activation may assist the kidney in metabolic conditions such as diabetes mellitus. Hasegawa et al. demonstrated that reduced SIRT1 in the proximal tubules represents the initiation of diabetic nephropathy using animal models of diabetes mellitus. Additionally, SIRT1 is implicated in the pathogenesis of diabetic nephropathy (Hao, 2015). Sirtuin 1 (Sirt1) a NAD+-dependent protein deacetylase, participates in various physiological activities, including hypoxia stress, DNA repair, cell aging, inflammatory, and mitochondria regulation; yet, its degradation is required for the formation of ND. SIRT1 expression was significantly decreased in the renal of diabetic db/db rats in previous research. Recent research, however, indicates that SIRT1 is involved in the endoplasmic reticulum stress response to hyperglycemia and hypoxia (Zhang et al., 2021).

B. The Expression of TNF-α by Immunohistochemistry in Kidneys tissues

The expressions of TNF-α, appeared brown on the IHC staining. The staining pattern was mainly in the form of cytoplasmic staining. The proportion of epithelial cells with positive intracytoplasmic brown staining was calculated, then compared to all epithelial cells per field of view; the staining intensity was reported in 4 intensity levels (negative, weak, moderate, and strong).

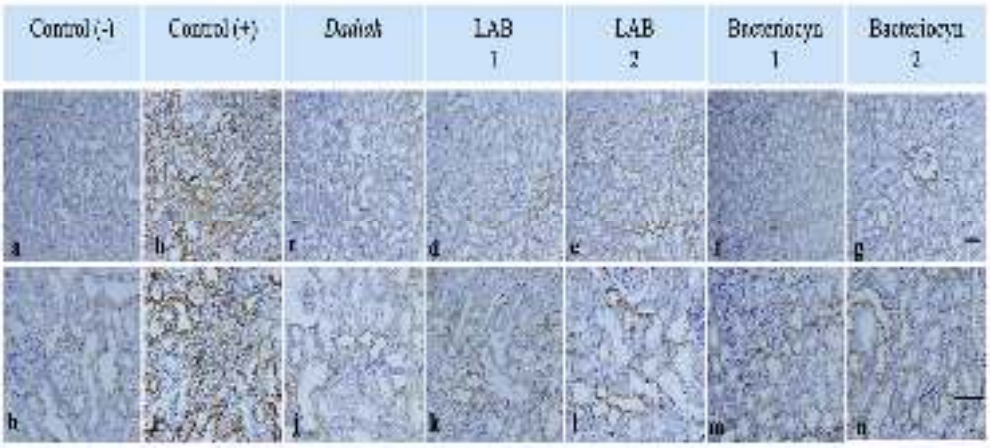

Figure 5 The assessment of TNF-α expression with immunohistochemistry

The staining of TNF-α immunohistochemistry in the kidney tissue of animal model; the negative control group (a, h) and the positive control (b, i), the treatment with dadiah (c, j), the low-dosage lactic acid bacteria (d, k) and the high dosage (e, l), and the low-dosage bacteriocin (f, m) and the high dose (b, i). The TNF-α was stained brown in some tubular epithelial cells and some cells in the stroma, with a weak staining in the matrix around the glomeruli and tubules. There was an increase in the TNF-α expression in the alloxan induction group, both in epithelial and stromal cells. The administration of dadiah, lactic acid bacteria, and bacteriocin, showed lower TNF- α expression than the positive control.

Table 5 The average number of TNF expression in each treatment

Sampel	Average	Standard Deviation	Notation
C–	16.6667	5.16398	a
C+	76.6667	5.16398	d
P1	20.0000	6.32456	ab
P2	23.3333	12.11060	abc
P3	30.0000	0.00000	c
P4	26.6667	8.16497	bc
P5	23.3333	10.32796	abc
Chi-square count	= 24.362		
p-value	= 0.000		

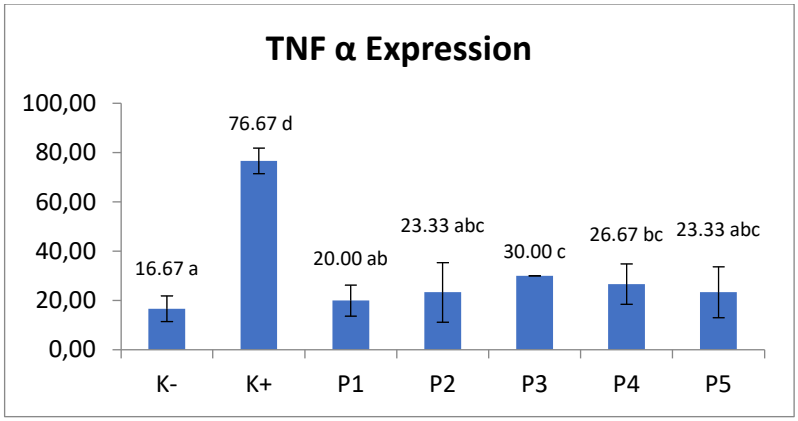

Figure 6 The Graph of TNF-α expression numbers in each treatment

Based on figure 6, it can be seen that the highest average of TNF expression in the C+ group (induced by alloxan + proteinuria) was 76.67±5.16, and the lowest average of TNF expression was in the C – group (not induced by alloxan and not given any treatment), which was equal to 16.67±5.16. To prove whether there was a statistically significant difference in the average number of TNF expression, the Kruskal Wallis statistical analysis would be carried out.

Based on the results of the Kruskal Wallis test, the p-value was smaller than (0.000 < 0.050), so it can be concluded that there is a significant difference in the average TNF expression number between treatments. To see the difference, further tests were carried out using the Mann Whitney test with the results notation in table 5. It can be seen that: The highest average of TNF expression in the C+ treatment was significantly different from the C-, P1, P2, P3, P4, and P5 treatments. The lowest average of TNF expression in C- groups was significantly different from C+, P3, and P4 treatment groups, but C- groups was not significantly different from P1, P2, and P5 treatment groups.

Additionally, scientific data indicates that the inflammatory factors tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) are well reported to contribute to renal impairment in diabetes (Song et al., 2020). Probiotics appear to reduce inflammation and oxidative stress markers, according to a growing body of studies (Mahmoodpoor et al., 2017). Diabetes and obesity are both metabolism disorders associated with a low-grade inflammatory state. Tumour Necrosis Factor alpha (TNF-α) is a marker inflammation cytokine that has been shown to phosphorylate the insulin receptor's serine residue substrate (IRS-1), inactivating it, while IL-1, TNF-α, and IFN are known to function synergistically by invading the pancreas and generating-cell damage and apoptosis (Maciel et al., 2020; Miraghajani, 2017; Sharma et al., 2018). In STZ-induced

diabetic rats, Lactobacillus casei strain Shirota significantly reduced pro-inflammatory cytokines Interleukin-6, Interleukin-4, and CRP. In a diabetes kidney rat model fed for 12 weeks, a probiotic mixture decreased TNF-α and increased IL-10. Similar studies have demonstrated the anti-inflammatory benefits of probiotic lactobacilli. Thus, our findings corroborate previous reports indicating that L. fermentum spp. had anti-inflammatory properties (Archer et al., 2021).

C. The Deposition of fibro-collagen matrix with HE Sirius red (Interstitial Fibrosis)

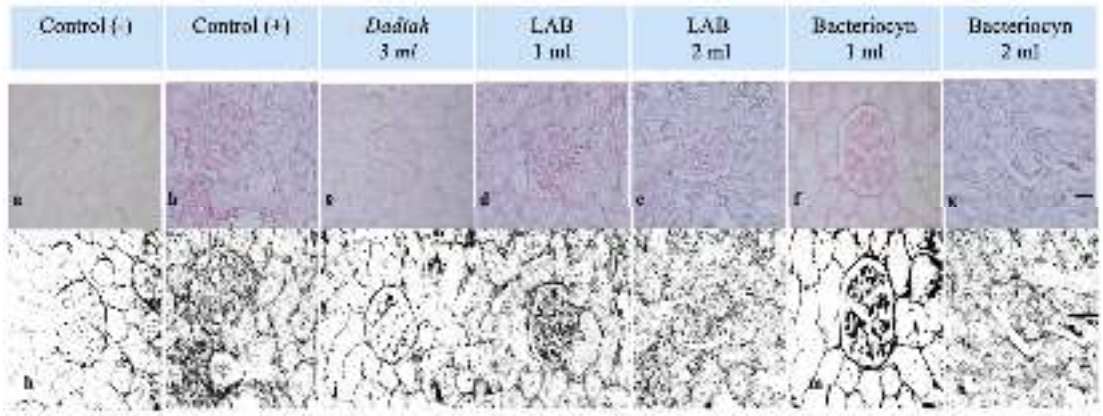

Figure 7. Deposition of fibro-collagen matrix in kidneys with Sirius Red staining

The connective tissue staining of the experimental animal kidneys with Sirius Red stained showed the interstitial and periglomerular connective tissue. The connective tissue matrix was stained with magenta. Negative control group (a, h), positive control (b, i), treatment with *dadiah* (c, q), low-dose lactic acid bacteria (d, k), high-dose (e, l), low-dose bacteriocin (f, m) and high-dose (b, i). The collagen deposition was measured using the ImageG program by extracting the red area, converting the image to black and white, and measuring the percentage area of the coloured area per unit area. The collagen deposition was lower in the experimental animals with *dadiah* treatment, lactic acid bacteria, and bacteriocin treatment, compared with the positive controls. The lowest collagen deposition was in the *dadiah* treatment, compared to other treatments. Induction with alloxan administration showed an increase in collagen matrix deposition in the renal parenchyma as a sign of glomerulosclerosis. (Lan, 2011; Navarro & Mora-ferna, 2006)

Table 6 Average of Fibrosis Interstitial Fibrosis in The Groups

Samples	Average	Standard Deviation	Notation
Negative Control (C-)	12.0667	0.78145	a
Positive Control (C+)	17.6667	0.90480	c
P1	14.9333	1.50687	b
P2	15.2833	1.95900	b
P3	15.8167	1.98133	b
P4	15.1833	1.79490	b
P5	15.0667	1.18434	b
F count	= 7.117		
p-value	= 0.000		

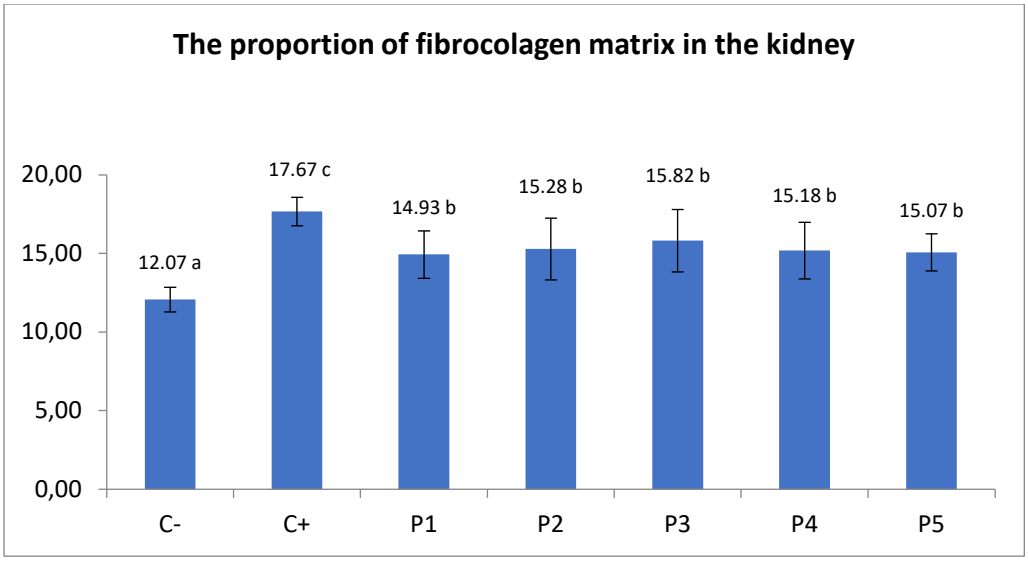

Figure 8 The proportion of fibrocolagen matrix in the kidney tissue

Based on figure 8, it can be seen that the highest average of Glomerular fibrosis rate in the C+ treatment (induced by alloxan + proteinuria) was 17.67 ± 0.90, and the lowest average of Glomerular fibrosis was in the C– treatment (not induced by alloxan and not given a treatment), namely of 12.07 ± 0.78. The one-way ANOVA statistical analysis would be used to determine how a statistically significant difference in the average number of Glomerular fibrosis existed. The one-way ANOVA test resulted in a p-value less than (0.000< 0.050), indicating a statistically significant difference in the average number of Interstitial Fibrosis between treatments. To demonstrate the distinction, more tests were conducted using the Duncan test and the notation results in table 5.4. It can be seen that: The highest average of Glomerular fibrosis in the C+ treatment was

significantly different from the C-, P1, P2, P3, P4, and P5 treatments. The lowest average of kidney fibrosis in the C- treatment was significantly different from the C+, P1, P2, P3, P4, and P5 treatments.

The activation of metabolic, inflammatory, and hemodynamic pathways describes the pathophysiology of DKD. For example, chronic hyperglycaemia leads to increased protein kinase C (PKC) activity, alterations in polyol metabolism, increased secretion of profibrotic cytokines (such as TGF-B1), and non-enzymatic glycosylation glycation of glomerulosclerosis structures and the formation of AGEs. The accumulation of aberrant protein glycation markers is a significant factor in the onset and progression of DKD. AGEs increase in the mesangial and glomerular capillary walls in people with ND, according to immunohistochemistry findings. The kidney plays a crucial role in AGEs metabolism (Macisaac et al., 2014). Renal fibrosis, myofibroblast, podocyte dysfunction, basement membrane thickening, and extracellular matrix (ECM) protein build-up are all regarded to be standard features of ND. Podocytes are a type of high differentiation glomerular epithelial cell that has been linked to the early pathogenic mechanism of ND pathogenesis (Gondaliya et al., 2020; Ishii et al., 2020; Y. Li et al., 2019; Wu et al., 2020; Yao et al., 2018). Furthermore, the increase in inflammation directly destroys renal function (Y. Li et al., 2019). In diabetes, the deposition of advanced glycation end products (AGEs) plays a crucial role in the development of ND. Additionally, inflammation and peroxidation are associated with the onset and progression of ND, respectively. The main characteristics of DN include a thick basement membrane, mesangial expansion, podocyte loss in the glomerular, and increased urine microalbumin excretion. Further, extracellular matrix (ECM) protein build-up plays a vital role in developing DN. Diabetic nephropathy is also characterized by renal fibrosis and glomerular sclerosis (Kundu et al., 2020).

CONCLUSION

Oral administration of *dadiah* and probiotics and secondary metabolite compounds of lactic acid bacteria have been shown to increase the production of sirtuin-1 and reducing the TNF-α expression that marker in stress oxidative and inflammatory processes caused by hyperglycaemia, and therefore alleviating renal fibrosis.

Administration of *dadiah* solution, isolate probiotic strain L fermentum, and isolate bacteriocin from *dadiah* has been shown to ameliorate renal tissue fibrosis in diabetic

nephropathy mice when stained with Sirius-red. In addition, oral administration of *dadiah*, probiotics and secondary metabolite compounds of lactic acid bacteria showed to increase the expression of sirtuin-1 and SOD, which functions were to reduce stress oxidative and inflammatory processes caused by hyperglycaemia, and therefore alleviating renal fibrosis.

The findings of this study could be to develop novel treatments for ND that aim to reduce the cascade of oxidative stress and inflammatory signals in kidney tissue.

Authors Contributions

All researchers have contributed both in the preparation of research proposals, laboratory research and the preparation of manuscripts to be published in journals.

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Conflict of interest

The authors declare no conflict of interest

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