DOI: 10.7860/JCDR/2024/69264.19474

Research Protocol

Biochemistry Section

The Effect of Probiotic Supplementation on the Levels of Biomarkers in Diabetic Animal Model: A Research Protocol

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ABSTRACT

Introduction: Type 2 Diabetes Mellitus (T2DM) is characterised by hyperglycaemia, insulin secretion defects, or resistance. Most T2DM drugs help to improve glycaemic status, but the response varies among individuals. The modern lifestyle with unhealthy eating habits leads to gut dysbiosis. Altered gut microbiota can disrupt the host's metabolic and signaling pathways, intestinal barrier integrity, and function. Probiotics could restore a healthy microbiota in the intestine, thus improving glycaemic status.

Need of the study: Probiotics may assist in re-establishing a healthy microbiota composition in the intestine. Limited studies have evaluated the supplementation of probiotics for effectively managing T2DM.

Aim: To investigate the effects of probiotics on biomarker levels in type 2 diabetic male Wistar rats.

Materials and Methods: This interventional case-control study will be conducted at the Centre for Toxicology and Developmental Research (CEFTE) at Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India. The study will involve 46 male Wistar rats divided into five groups. Diabetes will be induced by feeding the animals a High-Fat Diet (HFD) and administering a low dose of Streptozotocin (STZ) injection. Groups 1 and 2 will be on basal and HFDs, serving as negative and positive controls, respectively. Groups 3, 4 and 5 will be the intervention groups. The study duration will be four weeks and three days for diabetes induction and six weeks for intervention. Blood samples will be collected periodically to assess biomarkers, and at the end of the study, internal organs will be harvested for histopathological examination. Institutional Ethics Committee (IEC) approval has been obtained. Categorical variables will be analysed using Chi-square or Fisher's exact test, while continuous variables will be analysed using repeated measures Analysis of Variance (ANOVA). A p-value of ≤0.05 will be considered statistically significant, and statistical analysis will be performed using Statistical Package for Social Sciences (SPSS) version 16.0.

Keywords: Gut dysbiosis, Hyperglycaemia, Intestine, Microbiota

INTRODUCTION

Diabetes mellitus is a chronic, irreversible metabolic disorder. In 2021, worldwide, 536.6 million adults had T2DM as per the International Diabetes Federation (IDF) Atlas; this makes up 10.5% of the population.This number is projected to increase to 783.2 million people by 2045 [1]. Globally, the number of Daily Adjusted Life Years (DALYs) due to T2DM was 66.3 million in 2019, showing a 27.6% increase since 1990 [2]. In the Southeast Asia region, there are currently 90 million diabetic individuals. This figure is expected to rise to 113 million by 2030 and further to 152 million in another 15 years. Additionally, Impaired Glucose Tolerance (IGT) affects 47 million adults, who are at risk of developing diabetes without effective preventive measures [3]. Anjana RM et al., studied that the prevalence of T2DM in India is around 7.3% [4]. The Indian Council of Medical Research (ICMR) guidelines suggest that diabetes rates should be kept below 7% [5]. According to Joshi SR the mean Glycated Haemoglobin (HbA1c) in India is 9.0%, which exceeds global standards. Many diabetics go undetected and present with complications at the time of their T2DM diagnosis [6].

Diabetes mellitus is a complex disorder with varied pathogenesis. Wide combination of drugs has been tried depending on the presence of associated co-morbidities. Despite multiple drug therapies, a larger number of diabetics remain to have poor glycaemic control. Thus, current advances in drug regimens could alter the biochemical levels only minimally. Long standing diabetic individuals are fraught with anxiety and dismay [7]. T2DM can lead to irreversible end-organ dysfunctions. Three main factors that increase a person's risk of developing T2DM are a sedentary lifestyle, a low-fiber HFD, and genetic factors. The treatment of T2DM commonly involves diet modification, exercise, hypoglycaemic drugs, and lipid-lowering medications. Diabetes mellitus cannot be reversed once it has been established for a long time. Probiotics have been found to reverse gut dysbiosis and are therefore crucial in managing resistant cases of diabetes mellitus [8].

The human gut microbiota includes bacteria, viruses, archaea, fungi, and protozoa; they interact with the host in complex ways. These microorganisms play important roles in digestion, nutrient absorption, and immune function [9]. Bacteria are the most abundant microorganisms among the human gut microbiota, accounting for 99% of the total population [10]. The four primary phyla of bacteria in the human gut are Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria. Firmicutes, which are gram-positive bacteria, constitute about 64% of the total colonic microbiota. Bacteroidetes, which are gram-negative bacteria, account for approximately 23%. Proteobacteria and Actinobacteria make up roughly 8% and 3% of the total microbiota respectively [11]. Gut bacteria play beneficial roles in human health by contributing to the digestion and absorption of complex polysaccharides and dietary fiber due to the presence of the required enzymes in the bacteria. These compounds are further metabolised into Short-Chain Fatty Acids (SCFAs) such as butyric, acetic, and propionic acids [12,13]. SCFAs release energy that could regulate gut health by promoting the growth of beneficial bacteria, maintaining intestinal permeability and immune function, and also exhibiting anti-inflammatory effects [14]. They also produce essential vitamins, such as vitamin K and vitamin B12 [15,16].

The gut dysbiosis in T2DM consists of a decreased number of beneficial gram-positive bacteria such as Firmicutes, and an increased number of gram-negative bacteria, such as Bacteroidetes,

and Proteobacteria [17]. This shift in the composition of bacteria in T2DM patients may lead to increased intestinal permeability, Insulin Resistance (IR), and low-grade inflammation, contributing to the adverse prognosis of T2DM with the onset of complications involving multiple organs [18]. Microbe-derived compounds are believed to interact with receptors on epithelial, hepatic, and cardiac cells, to modify host physiology that is directly or indirectly linked to the progression of IR in T2DM patients [19]. Therefore, altering the gut microbiota through dietary probiotic supplementation will be a promising treatment option for T2DM. This protocol will be aimed at studying the effect of probiotics on the alterations of levels of biomarkers in diabetic male Wistar rats.

Primary objectives:

1. To compare blood glucose, insulin, and lipid profile in diabetic rats before and after intervention with probiotics.
2. To compare inflammatory markers such as C-Reactive Protein (CRP), Interleukin-6 (IL-6), and Tumour Necrosis Factor-alpha (TNF-α) in diabetic rats before and after intervention with probiotics.
3. To compare markers of endotoxemia such as Lipopolysaccharide (LPS), Lipopolysaccharide-Binding Protein (LBP), occludin-2, and CD14 before and after intervention with probiotics.
4. To compare histopathological studies of the pancreas, kidney, intestine, and liver across the groups.

Null hypothesis: Supplementation of probiotics does not alter the levels of biomarkers in diabetic male Wistar rats.

Alternative hypothesis: Supplementation of probiotics alters the levels of biomarkers in diabetic male Wistar rats.

REVIEW OF LITERATURE

Several studies have evaluated the antidiabetic effects of probiotic strains in animal models. According to Memarrast F et al., probiotics containing *Lactobacillus reuteri, Lactobacillus crispatus*, and *Bacillus subtilis* have been found to show beneficial effects on the Oral Glucose Tolerance Test (OGTT), insulin, HbA1c levels, and lipid profile in STZ-induced diabetic rat models [20]. There was a significant increase in Glucose Transporter 4 (GLUT4) and Peroxisome Proliferator-Activated Receptor-gamma (PPAR-γ) expression in the adipose tissue of rats fed probiotics compared to controls [20]. Feeding STZ-induced diabetic mice with *Lactobacillus delbrueckii* led to a decrease in fasting blood glucose and fetuin-A levels, as well as an increase in serum sestrin 3 levels [21]. In the study by Hsieh PS et al., probiotic strains such as *Lactobacillus salivarius, Lactobacillus johnsonii, Lactobacillus reuteri*, and *Bifidobacterium animalis* are the major components of the probiotic product. The probiotic effects were evaluated in a type-2 diabetic rat model induced by STZ treatment and a HFD. Injections of low-dose STZ at 10-20 mg/kg body weight with a high-energy diet successfully induced hyperglycaemia and caused damage to the beta cells of the pancreas. The probiotic alleviated the symptoms of type-2 diabetes in STZ-treated rats by protecting the function of β cells and stabilising glycaemic levels [22]. Consumption of a HFD causes dysbiosis of the intestinal mucosal milieu, compromising the integrity of the mucosal barrier. TNF-α is a proinflammatory cytokine that, when elevated, is known to phosphorylate the serine residue of Insulin Receptor Substrate-1 (IRS-1), causing its inactivation. Interleukin-1 beta (IL-1β), TNF-α, and Interferon-gamma (IFN-γ) are known to act synergistically by infiltrating the pancreas and inducing β-cell damage and apoptosis, thus inducing IR. Supplementation of *Lactobacillus fermentum* shows downregulation of these genes in the intestine, liver, adipose tissue, and skeletal muscle primarily by stimulating the expression of the anti-inflammatory regulatory cytokine IL-10. *Lactobacillus fermentum* has the ability to reduce gut LPS levels and improve barrier function. The enhanced barrier functionality is evident by the enhanced expression of zonulin-1 (ZO-1). It also augments the expression of Glucagon-Like Peptide 1 (GLP-1) in the intestine and GLUT4 expression in adipose tissue and skeletal muscle, thereby improving inflammation and IR. Additionally, it reduces the invasion of inflammatory cells in the intestine [23]. These results suggest that probiotic-containing diets might be useful in the earlier control of blood glucose levels in diabetic patients.

MATERIALS AND METHODS

The interventional case-control study involves 46 male Wistar rats. The study will be conducted at the CEFTE, which is an independent unit of Sri Ramachandra Institute of Higher Education and Research (SRIHER), a Deemed University. CEFTE is a Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) registered and OECD-GLP certified Test Facility.

Ethics statement: The experimental protocol is approved by the Institutional Animal Ethical Committee (IAEC) of Sri Ramachandra Institute of Higher Education and Research (SRIHER); the ethical approval number is IAEC/69/SRIHER/823/2023. The experiments will be performed according to the norms of the CPCSEA The sample size of the study is determined using Federer's formula. The number of samples included in Group 1, which will serve as a negative control, is six. Considering attrition in diabetic animal models with or without intervention, the sample size in each of groups 2 to 5 is 10. Therefore, the sample size for the study is 46 [24,25].

Inclusion criteria: Male Wistar rats, age 8-10 weeks, weight 200±20 g.

Exclusion criteria: Female Wistar rats.

The male Wistar rats will be grouped into five groups: group 1 consists of healthy rats (negative control), while group 2 will serve as the diabetic positive control. Groups-3-5 (G3,G4,G5) will be the intervention groups. The STZ-induced diabetes model may result in an increase in mortality (25-30%); therefore, in the diabetic groups (groups-2-5), 10 rats will be included for the study. Female Wistar rats will not be included in the study, as they are prone to hormonal fluctuations during the female fertility cycle [26], which could falsely alter the levels of biomarkers and lead to inconsistent data.

The duration of the study will be four weeks of induction of diabetes mellitus followed by six weeks of intervention. Forty Wistar rats (groups-2-5) will undergo induction of type 2 diabetes mellitus by feeding them a HFD for four weeks, followed by a low dose of STZ intraperitoneal injection. The animals will exhibit features of diabetes mellitus three days after receiving STZ. The induction of diabetes with a combination of a HFD and low-dose STZ has been found to be an effective type 2 diabetic animal model [Table/Fig-1].

<table>
<tr><td rowspan="7">Acclimatization 5 days (n= 46 male Wistar rats)</td><td></td><td rowspan="2">Induction of diabetes mellitus</td><td colspan="3">Groups</td><td rowspan="2">Intervention</td><td rowspan="2">Day 1</td><td rowspan="2">Third week</td><td rowspan="2">Sixth week</td></tr>
<tr><td colspan="3">Normal/ Diabetic rats</td></tr>
<tr><td>6 rats</td><td>Basal diet</td><td>G1</td><td>Normal Healthy rats (n=6)</td><td>Basal diet</td><td rowspan="5">Blood sample collection</td><td rowspan="5">Blood sample collection</td><td rowspan="5">Blood sample collection</td></tr>
<tr><td rowspan="4">40 rats</td><td rowspan="4">High-fat diet</td><td>G2</td><td>Diabetic rats (n=10)</td><td rowspan="4">Intraperitoneal streptozotocin</td><td>Basal diet</td></tr>
<tr><td>G3</td><td>Diabetic rats (n=10)</td><td>Probiotic yogurt & Basal diet</td></tr>
<tr><td>G4</td><td>Diabetic rats (n=10)</td><td>Bifilac & Basal diet</td></tr>
<tr><td>G5</td><td>Diabetic rats (n=10)</td><td>Metformin & Basal diet</td></tr>
<tr><td colspan="4">Duration: 4 weeks</td><td>3 days</td><td colspan="4">Duration : 6 Weeks</td></tr>
</table>

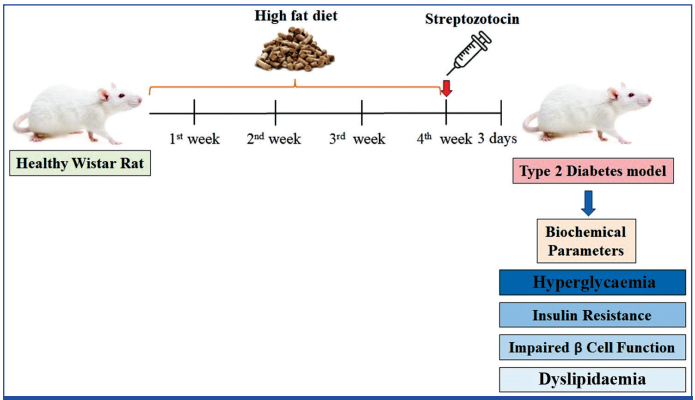
[Table/Fig-1]: Shows the study groups and timeline of the study protocol.

Animal Housing and Care

The animals will be housed in polypropylene cages equipped with stainless steel grid tops, with three rats accommodated in each cage. The combined weight of rats in a single cage will not exceed 1 kg. Each rat will be individually identified by marking their tails with a permanent marker. The bedding material, which will consist

of autoclaved paddy husk, will be replaced every other day. The environmental temperature will be maintained between 19 and 23 degrees celsius, with humidity levels between 30 and 70 percent, and with 12 to 15 air changes per hour. Each day, the animals will be subjected to 12 hours of artificial light and 12 hours of darkness. Autoclaved bottles containing potable UV-treated water will be made available at all times. Water bottles will be replaced daily, and food will be available at all times to ensure the well-being of the rats.

Development of High-Fat Diet (HFD) and Streptozotocin (STZ)-induced type 2 diabetic rat model (Groups 2-5): After acclimatization, 40 Wistar rats (groups 2-5) will be fed a HFD for four weeks. The use of a HFD for the induction of T2DM in rats will be conducted according to the study by Kadir NAA et al.,. The nutrient composition of the HFD includes 40% fat, 43% carbohydrates, and 17% proteins, vitamins, and minerals [27]. At the end of the four weeks, the rats will be fasted for six hours before receiving an intraperitoneal injection of STZ at a dose of 30-35 mg/kg body weight [28]. Three days after the STZ injection, blood samples will be collected from the tail vein for the analysis of fasting serum insulin and blood glucose levels. Animals exhibiting blood glucose levels above 200 mg/dL, along with clinical signs such as polyuria and polydipsia, will be considered diabetic rats. Homeostatic Model Assessment-Insulin Resistance (HOMA-IR), Quantitative Insulin Sensitivity Check Index (QUICKI), and HOMA-β cell function will be derived [Table/Fig-2].



[Table/Fig-2]: Shows the procedure for the development of the diabetic animal model.

Probiotic Diet Intervention

Three types of intervention will be performed in three groups (groups 3-5), with 10 animals in each group. The remaining 10 animals (group 2) will not undergo any intervention and will serve as positive diabetic controls. The interventions will consist of plain probiotic yogurt, bifilac, or metformin in groups 3 to 5, respectively. The test items will be orally administered through oral gavage, mimicking the oral administration of diet and antidiabetic drugs in humans.

The basal diet will be obtained commercially from an approved vendor, with each rat consuming about 15-20 g of basal diet per day. Commercial plain probiotic yogurt with a minimum fat content of 4% will be obtained daily from a dairy vendor. Bifilac contains *Lactobacillus sporogenes* (50.0 M), *Streptococcus faecalis* (30.0 M), *Clostridium butyricum* (2.0 M), and *Bacillus mesentericus* (1.0 M). An adult human dose of bifilac is 1.5 g per day, equivalent to 21.42 mg/kg [29]. The human dose is converted to a rat dose by multiplying by 6.1 (body surface area conversion factor- human dose to rat dose) to obtain 128.5 mg/kg, which will be rounded off to 150 mg/kg for treatment [30]. Metformin is an oral antidiabetic drug typically prescribed at doses ranging from 500 to 2550 mg per day, depending on the glycaemic response of the diabetic individual. The rat equivalent dose will be calculated from the human dose as 100 mg/kg [31]. Bifilac and metformin will be obtained from SRIHER after obtaining the necessary approvals. Any opened yogurt containers, leftovers of bifilac, and metformin will be discarded.

Quality standard analysis of yogurt:

The yogurt will undergo the following analyses:

- Molecular analysis of the probiotic bacterial strain
- Microbiological analysis of the probiotic bacterial strain
- Physical/sensory analysis of the yogurt: The sensory attributes, including taste, texture, aroma, and appearance, will be evaluated [32].
- Chemical analysis of the yogurt: This includes proximate analysis, nutritional analysis, pH measurement, total solids, titratable acidity, fat content, dry matter, solid non fat, and free fatty acid. Chemical analysis will be conducted according to the Association of Official and Analytical Chemists (AOAC) guidelines [33].

Monitoring of rats: Control and experimental rats will be weighed on day zero and at weekly intervals during the study period. Changes in food consumption, calorie intake, and water consumption of the rats in all groups will be monitored daily. Additionally, the general appearance, behaviour, clinical signs, mortality, and morbidity of the rats will be monitored.

Safety considerations: The safety and wellbeing of the animals involved in this study are of paramount importance. Ethical guidelines and established standards for animal research will be strictly followed throughout the study.

Anthropometric analysis: Body weight, abdominal circumference, thoracic circumference, and the ratio of abdominal circumference to thoracic circumference will be measured [34]. BMI and Lee index will be calculated using the formulas shown below:

BMI=weight(g)/length(cm))^2; Obesity threshold: BMI>0.687g/cm^2

Lee index=(weight(g)/lengthy(cm)) ^ (1/3); Obesity threshold: Lee index >310g

Procedure for collection of blood and harvesting organs from the abdomen: Blood samples will be collected from the retroorbital vein at the beginning of the intervention and at the end of the third week. At the end of the experiment (sixth week), animals will be sacrificed, and blood samples will be collected from the abdominal aorta. Internal organs such as the liver, pancreas, intestine, and kidney will be harvested for histopathological analysis. The distribution of fat will also be measured.

Outcomes-analysis of biomarkers: Biochemical analysis will be performed at baseline and at three weeks and six weeks post-intervention period for all the rats belonging to the five groups (groups 1 to 5). Biochemical parameters such as blood glucose and lipid profile will be analysed by standard methods. Inflammatory markers such as CRP, IL-6, and TNF-α, and markers of endotoxemia such as LPS, LBP, occludin-2, CD14, and insulin will be analysed by ELISA. HOMA-IR, HOMA-β cell function, and QUICKI will be calculated. Histopathological findings in the pancreas, kidney, intestine, and liver, as well as with the extent of distribution of fat in the peritoneum and retroperitoneal regions will be compared among different groups.

STATISTICAL ANALYSIS

Statistical analysis will be conducted using SPSS version 16.0. Categorical variables will be analysed by Chi-square or Fisher's-exact test. Continuous variables will be analysed by repeated measures ANOVA. Post-hoc analysis using the Least Significant Difference (LSD) test will be performed. A p-value <0.05 will be considered statistically significant.

Authors contributions: TS and SS: conceptualisation, SS, EB, VG, and PM: methodology, TS and SS: resources, TS, SS, EB, VG, PM: original draft preparation, EB and SS: review and editing, SS, VG and EB: supervision, TS, SS and VG: project administration, TS and SS: funding acquisition. All authors have read and agreed to the final version of the manuscript.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]

- Plagiarism X-checker: Dec 25, 2023
- Manual Googling: Feb 13, 2024
- iThenticate Software: Apr 29, 2024 (11%)

ETYMOLOGY: Author Origin

EMENDATIONS: 7

AUTHOR DECLARATION:

- Financial or Other Competing Interests: The project is funded by the Sri Ramachandra Institute of Higher Education and Research through Founder-Chancellor Shri. N.P.V. Ramasamy Udayar Research Fellowship (Grant No. U02M210724).
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? No
- For any images presented appropriate consent has been obtained from the subjects. No

Date of Submission: **Dec 25, 2023**
Date of Peer Review: **Jan 29, 2024**
Date of Acceptance: **May 01, 2024**
Date of Publishing: **Jun 01, 2024**