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Evaluation of the protective effect of probiotics against alloxan induced diabetes in male rats

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ABSTRACT Environmental and genetic factors are two of the many contributing factors to diabetes. Many studies shows that the molecular processes of insulin resistance are connected to elevated oxidative and or inflammatory factors. The gut microbiota, combined with various environmental influences and predisposed genetic features, plays a part in the development of diabetes. Moreover, it has been suggested that changes in the gut microbiota can cause a mucosal immune response and increase intestinal permeability, both of which are factors in diabetes. Probiotics have been found to be an effective adjuvant in treatments for insulin resistance and may help maintain a better gut microbiota, the objective of the study is to assess the possible protective effect of probiotics on alloxan induced diabetes in male rat. Atotal of 18 matured Normoglycemic male Sprague Dawley rats, were randomly assigned in to 3 groups (6 in each group), Normal group receiving saline vehicle orally, Induced (Alloxane) group injected with alloxan (120 mg/kg i.p.) and Probiotic treated group treated with probiotics (2.5×10^9 CFU, 160 mg) for 15 days before and 5 days after alloxan injection. Animals with fasting blood glucose levels >250 mg/dL consider diabetes. Blood samples were collected for biochemical analysis (e.g., glucose, insulin, oxidative stress markers). Data were analysed using ANOVA and Kruskal-Wallis tests, with significance set at $p < 0.05$. The results revealed significantly higher serum level of glucose, reduced insulin and c-peptide in alloxan induced diabetes rats compared to normal group. In addition, there are significant improvements in antioxidant system (SOD and GSH), a lower level of lipid peroxidation marker (MDA) as well as lower levels of serum concentration of urea and creatinine in probiotic-treated group. Probiotics may offer a promising therapeutic effect in improving diabetes complication and restore pancreatic B cell function, prevent cell distraction via gut microbiota modulation, restore intestinal permeability and decrease oxidative stress and markers of inflammation.

Key word: diabetes mellitus, oxidative stress, probiotic, antioxidant activity

I. Introduction

Diabetes mellitus (DM) is a persistent metabolic illness characterized by high blood glucose caused by deficiency or resistance to insulin leading to numerous organ injury causing various complications such as diabetic kidney disease (DKD), diabetic retinopathy (DR), and diabetic cardiovascular disorders. Diabetes is classified into two types: type 1 and type 2 and gestational diabetes (GD**)**.[1] Oxidative stress results from a disproportion between reactive oxygen species (ROS) production and antioxidant defence mechanism and this possibly contributes to the pathogenesis of diabetes and its complications,[2] adults microbiota consists of 1,000–1,150 different bacterial species, include *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*,[3] with suggested potential health effects in gut microbiota modification and the release of metabolites, such as tryptophan and short chain fatty acids (SCFA). Probiotics are live

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bacteria that exert beneficial health effects when administered in sufficient amounts. The frequent probiotic species are Bifidobacterium and Lactobacillus genera, the less common probiotics are *Faecalibacterium prausnitzii, Akkermansia uciniphila, Streptococcus thermophilus, Saccharomycesbourlardii* and *Lactococcus lactis.*[4] Metabolic activities of probiotic bacteria may have shown the anti-oxidative effect through the scavenging of oxidants, the production of bioactive peptides has been recognized as an effective way to enhance anti-oxidative activity in foods that contain probiotic bacteria.[5] Additionally, the intestinal microflora contributes to various enzymatic activities that aid in transforming dietary compounds, thereby increasing the bioavailability of dietary antioxidants.[6] Probiotics release antioxidant compounds that primarily act as free radical scavengers, such as glutathione. They also produce extracellular polysaccharides (EPS), which demonstrate in vitro antioxidant and free radical scavenging activities and to the exhibition of metal chelating activity.[7] Some probiotics like Lactobacillus (Lb) fermentum ME-3 possessed substantial and a high anti-oxidative activity expressed manganese superoxide dismutase, can effectively eliminate hydroxyl radicals and contained the complete glutathione system (GR, GPx) necessary for glutathione recycling, transporting and synthesis.[8]

II. Materials and method

Animals

A total of 18 adult male Sprague Dawley rats were divided into 3 groups each group encompass of 6 individual male rats (n=6) weighing (150- 300 g) and with (12–16 weeks of age), housed in the animal house at the Faculty of Science University of Kufa. The animals were housed in an isolated room in group caging system, with temperature (18-26˚C) and humidity set at (30-70%). In a controlled atmosphere of 10 h light/14h dark cycles and was fed with standard laboratory diet and allowed to drink water ad libitum.

Study design

Groups divided as follow:

1) *Normal group* (n=6): rats did not receive any treatment, only 1ml normal saline 0.9% orally for 20 days.

2) *Diabetic Induced group (Alloxane) group* (n=6): Rats received a single dose of alloxan monohydrate (120 mg/kg) dissolved in 0.9% normal saline intraperitonially in order to induce diabetes.

3) *Treatment Group (probiotics group)* (n=6): each Rat received 1ml vehicle of normal saline 9% contain powder of blend of 10 strain of probiotics bacteria (2.5×10ˆ9) serving per colony (CPU) equivalent to (160) mg orally for 20 days, 15 days before alloxan injection and 5 days after alloxan injection.

Induction of diabetes mellitus

Diabetes was induced in fasting male rats 12 to 14 hours via a single intraperitoneal injection of 120 mg/kg of freshly prepared alloxan powder dissolved in 0.9% normal saline to ensure proper delivery,[9] on day 15 of study, fasting blood glucose was measured in the morning for each rat (as a baseline) before the induction of diabetes by injection of 1ml alloxan vehicle intraperitonially. After 48 hours of alloxan injection, rats exhibited significant hyperglycaemia characterized by fasting blood glucose levels more than 250 mg/dl, reaching 600 mg/dl. Subsequent measurement of fasting blood glucose was performed on days 16, 17, and 19 using (on-call plus) code chip (ACON laboratories, lnc, USA),[10] by directly flowing or gently massaging ('milking') the tail and collecting the blood on a glucose test strips.[11] At day 20, all rats were anesthetized using ketamine10%% (3 international units) combined with xylazine 2% (1 international unit) per 250 grams of body weight. The pancreas was immediately excised and fixed in 10% formalin for histopathological evaluation. The tissues were stained by H&E stain, the section is captured using a digital camera and light microscope at 40 X magnifier scale. Blood was obtained by directly flowing or gently to measure fasting blood glucose and the level of (Insulin, C-peptide, SOD, MDA, GSH, Urea and Creatinine) in serum of diabetic.

Statistical analysis

Analysis was conducted using Graph pad Prism version 8.1, presenting data as mean ± standard error means (SEM). One Way ANOVA was used for comparing

categories, followed by Bonferroni's multi-comparison test for post hoc analysis. Additionally, Kruskal-Wallis unidirectional non-parametric analysis was employed to assess histopathological changes among groups, with a significance level set at $p < 0.05$.

III. Results and Disscusion

At the end of the study results showed normal glucose level in normal group. In induced (Alloxan) group, the results revealed significantly higher level ($p<0.001$) of glucose compared to the normal group signifying Alloxan-induced diabetes. However, the probiotics group showed a significantly lower level of glucose compared to the induced group ($p<0.001$) with no a significant difference between the normal and probiotic group ($P = 0.5680$) (Fig. 1**)**

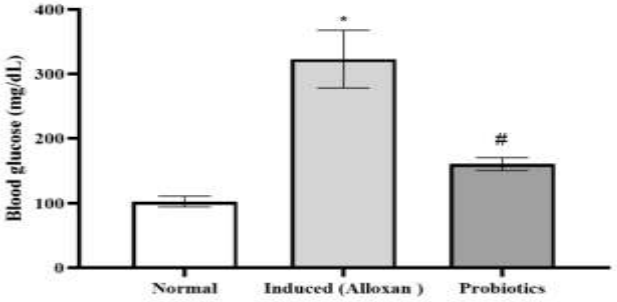

Fig. (1) Effect on glucose (mg/dl) level (n=6±SEM, *: significant when compared to the normal group, #: significant when compared to the induced group, One way ANOVA with Bonferroni post hoc test).

Regarding insulin, a significantly lower level was recoreded in the induced group compared to normal ($p<0.001$). However, those animals treated with probiotics showed a significantly higher level of insulin compared to the induced group ($p<0.001$) with no significant differences between the probiotic and the normal group ($p > 0.9999$) (Fig.2).

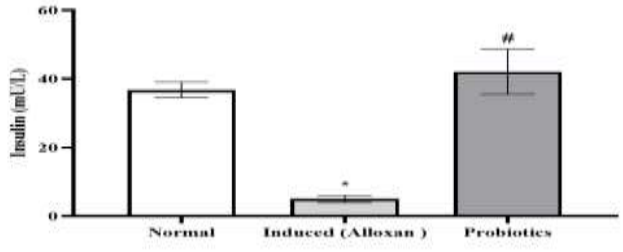

Fig. (2) Effect on insulin (mu/l) level (n=6±SEM, *: significant when compared to the normal group, #: significant when compared to the induced group, One way ANOVA with Bonferroni post hoc test).

In terms of C-peptide, similar to insulin where induction of diabetes in the Alloxan group resulted in significant depletion of C-peptide compared to the normal group ($p < 0.001$) while the treatment with probiotics resulted in significantly restoration of C-peptide compared to the induced group ($p < 0.001$) with no significant difference between the normal and probiotic group ($p > 0.9999$). (Fig.3).

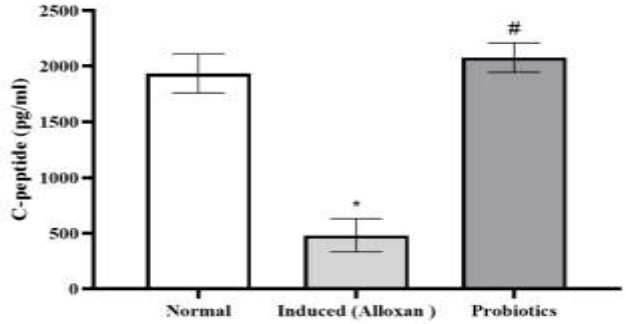

Fig. (3) Effect on c-peptide (pg/ml) level (n=6±SEM, *: significant when compared to the normal group, #: significant when compared to the induced group, One way ANOVA with Bonferroni post hoc test).

The levels of both antioxidant markers (SOD and GSH) were significantly reduced ($p<0.001$) in Alloxan treatment compared the normal animals with significantly restored antioxidant activity in the probiotic group ($p<0.001$). No significant differences were indicated between the normal group and the probiotic group in the level of SOD and GSH (Fig. 4 and 5, respectively) with ($p > 0.9999$) for both.

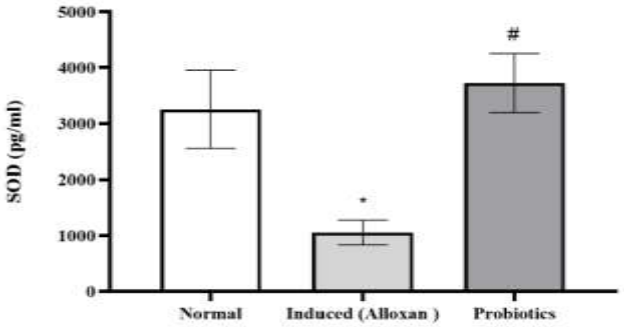

Fig. (4) Effect on SOD (pg/ml) level (n=6±SEM, *: significant when compared to the normal group, #: significant when compared to the induced group, One way ANOVA with Bonferroni post hoc test).

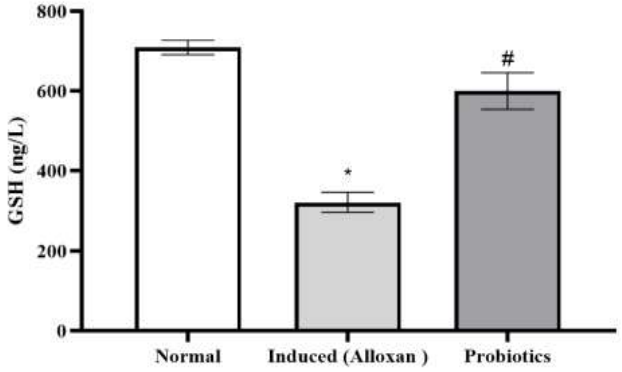

Fig. (5) Effect on GSH (ng/l) level (n=6±SEM, *: significant when compared to the normal group, #: significant when compared to the induced group, One way ANOVA with Bonferroni post hoc test).

A significantly higher level of the lipid peroxidation marker (MDA) was reported in the induced group compared to the normal group ($p<0.001$). However, the probiotic group showed significantly diminished oxidative stress ($p<0.05$) represented by the lower level of MDA. No significant differences were revealed between the probiotic group and the normal group ($p>0.9999$) (Fig. 6).

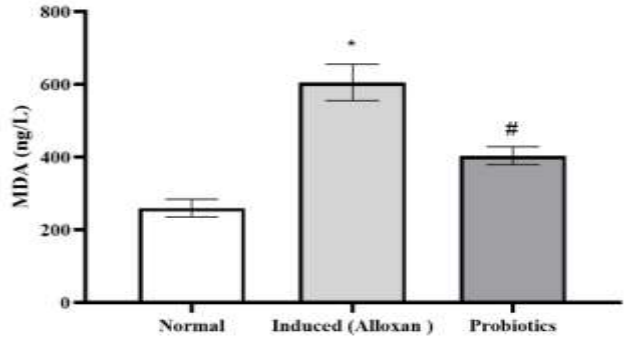

Fig. (6) Effect on MDA (ng/l) level (n=6±SEM, *: significant when compared to the normal group, #: significant when compared to the induced group, One way ANOVA with Bonferroni post hoc test).

The renal function test was also performed represented by serum urea and creatinine and showed that both markers are significantly higher in the induced group compared to the normal group (both with $p<0.001$) with no significant difference in the level of both urea and creatinine in the probiotic group when compared to the normal group (Fig. 7 and 8, respectively).

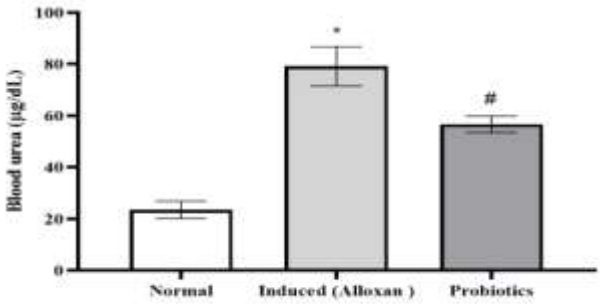

Fig. (7) Effect on urea (Mg/dl) level (n=6±SEM, *: significant when compared to the normal group, #: significant when compared to the induced group, One way ANOVA with Bonferroni post hoc test).

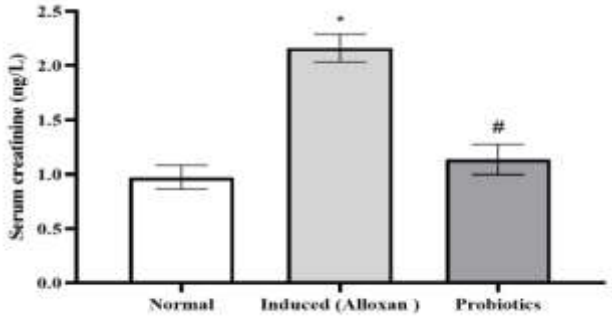

Fig. (8) Effect on creatinine (ng/l) level (n=6±SEM, *: significant when compared to the normal group, #: significant when compared to the induced group, One way ANOVA with Bonferroni post hoc test).

The histopathological changes are illustrated in figure 9 where normal appearance of the normal group (**A**) (black arrows) point to the pancreatic islet; Islets of Langerhans which appear normal, well-organized within the islet, suggesting functional insulin-producing beta cells and other endocrine cells. The red arrows indicate normal serous acinar cells. These exocrine cells are arranged in clusters and exhibit a typical cytoplasmic appearance, indicating active enzyme production (e.g., digestive enzymes like amylase and lipase) the cells preserve clear, intact nuclei and basophilic (blue-stained) cytoplasm, point to healthy zymogene cell with no signs of inflammation, necrosis, fibrosis, or vacuolation.

Significant alteration was reported in pancreatic structure and function from induced (alloxan) group (**B**). The section shows sever atrophied lesion in the pancreatic islet (black arrow) and moderate

degenerative changes in the serous acini and zymogene cells (Red arrows).

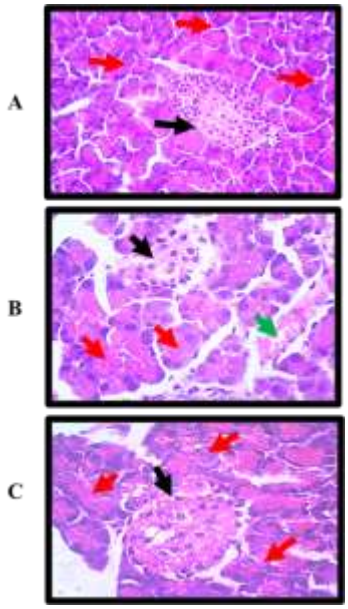

Fig. (9) Histopathological section of pancreas stained by H&E stain, the sections are captured using digital camera and light microscope at 20 X magnifier scale. A: normal group showing normal histopathological appearance. B: diabetic's male rat in induced (alloxan) group injected by Alloxan (120mg/kg) intraperitoneal. **C**: Probiotic group.

In the probiotics treatment group histopathological results suggested no significant alteration in pancreatic structure The section shows normal pancreatic islet (Black arrow) and mild degenerative changes in serous acini and zymogene cells (Red arrows) (**C**). Normal blood glucose levels; representative the baseline for well, non-diabetic conditions, was seen in normal group. However Induced group shows significantly elevated blood glucose levels compared to the Normal group represents hyperglycemia. Probiotics treatment significantly reduced blood glucose levels compared to the Induced (alloxan) group. Although, the levels are still higher than the Normal group, suggesting partial improvement. These findings agree with Meta-Analysis that evaluated multiple randomized controlled trials done to to assess the impact of probiotics on glycemic control[12], findings indicated that probiotic supplementation led to a significant reduction in fasting blood glucose levels among individuals with type 2 diabetes. Notably, trials utilizing multiple species of probiotics demonstrated more pronounced effects compared to those using a single species. Treatments with Probiotics significantly increased insulin levels In Induced (alloxan) groups, restoring them to levels comparable to the normal group and this agree with study that was made on STZ-induced diabetic rats treated with a 10-strain probiotic formulation (Lactobacillus and Bifidobacterium species) for 8 weeks. The study found that treatment with Multi-strain probiotics increased fasting insulin and C-peptide levels, Improved gut microbiota diversity and reduced markers of leaky gut, whereas ,Probiotics reduced endo toxemia by restoring gut barrier integrity, decreasing inflammation, enhancing beta-cell function and improve gut-pancreas signaling, leading to increased insulin secretion in diabetic rats[13]. The reduced C-peptide levels in the diabetic group point to impaired insulin secretion owing to β-cell dysfunction this is give emphasis to how prolonged hyperglycemia damages pancreatic β-cells. These results are agreed with study which confirmed that diabetic individuals typically show reduced C-peptide levels due to β-cell dysfunction.[14] Probiotics can improve gut microbiota and may facilitate better glucose metabolism. And these finding agreed with study which explain that probiotics can influence metabolic health positively and improves insulin sensitivity, leading to increased C-peptide level.[15] Treatment with Probiotics improves histological section of pancreas of male rats in normal group SOD levels compared to induced group. In induced Group the results Displays the lowest SOD levels, indicated oxidative stress or impaired antioxidant defense meachanism, these results are comparable with**,**[16] study that discuss oxidative stress as a main reason in reduced SOD activity in diabetic patients. Moreover,.Probiotics recognized to improve gut health and reduce oxidative stress, which might indirectly enhance SOD activity via Improvement in antioxidant defense, gut micro biota modulation and reduced inflammation. Glutathione (GSH) level explain highly significant decrease in induced group related to the severe oxidative stress associated with alloxan injection which obviously seen in induced (alloxan) group at the end of the study compared to normal group. In normal vs. Probiotics group there was No significant difference in GSH level demonstrating that probiotics effectively restore GSH to normal-control levels. Present study results agree with,[17] study which explains the Thiol groups present in glutathione and protein cysteine residues play a vital role in scavenging ROS. The -SH group in thiols reacts with ROS, neutralizing them and preventing cellular damage. Glutathione (GSH) is the most abundant non-enzymatic thiol in cells. It acts as a reducing agent, maintaining the redox state and

protecting cellular components from oxidative damage the partial reduction in MDA in probiotic group viewing probiotic s therapeutic effects in reducing oxidative damage.These finding agree with studies showed that probiotics can improve the antioxidant content of glutathione, superoxide dismutase, catalase, and glutathione peroxidase in diabetic rats by inhibiting lipid peroxidation, thereby reducing oxidative damage, increasing insulin secretion, reducing glycosylated hemoglobin level, and reducing intestinal absorption of glucose.[18] Prolonged hyperglycemia increases protein catabolism and urea production, leading to accumulation in the blood also oxidative stress arise from Ros activates inflammatory cytokines (e.g., IL-6, TNF-α), worsening kidney injury.[19] Probiotics significantly reduce urea levels compared to the induced group as seen from presents results and this agreed with,[20] study which demonstrating that Probiotics improve renal function by modulating gut microbiota, reducing uremic toxins (e.g., indoxyl sulfate) that exacerbate kidney dysfunction, Decrease systemic inflammation and oxidative stress that contribute to nephron damage,Restore gut-kidney axis function by enhancing gut permeability and reducing bacterial translocation. Creatinine is a product of muscle metabolism and is excreted by the kidneys. Elevated levels indicate renal dysfunction or reduced glomerular filtration rate (GFR). hyperglycemia damages renal tubules and glomeruli, reducing GFR and causing creatinine accumulation.This outcome aligns with findings that diabetic nephropathy leads to kidney damage and elevated blood creatinine Probiotics significantly reduce creatinine levels compared to the Diabetic group. Farther study[21] showing that the treatment with probiotics can improved kidney filtration and reduced systemic inflammatory markers (e.g. TNF-α). Observations from pesesnt histopathological sections illustrate the pancreatic islets (black arrow) appear normal in both sections. Adding, Both sections show mild degenerative changes in the serous acini and zymogenic cells (red arrows), suggesting an early response to treatment or stress adaptation. The mild degenerative changes visible in **(Fig.c)** indicate that probiotics could modulate inflammatory responses, providing some degree of cellular protection.**).**A study by,[22] specifically focused on the effects of a probiotic on diabetic and demonstrated that probiotics significantly mitigated pancreatic damage by reducing oxidative stress and inflammatory responses, highlighting their potential as a therapeutic adjunct in diabetes management.

V. CONCLUSION

Treatment with probiotics show possible protective effect in reducing damaging to pancreatic beta cell causing by oxidative stress mediators MDA and increase cellular anti-oxidants markers GSH and SOD and restoring levels of Glucose ,Insulin and C-peptide close to normal level via anti-oxidants ,anti-inflammatory and environmental gut microbiota modulation.

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