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Original Research Article

Evaluation of the Anti-Diabetic Potential of Probiotic *Lactobacillus fermentum* (PRI 29) Isolated from Cameroonian Fermented Cow Milk in Alloxan Induced Diabetes Type-1 Mice Model

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Abstract

Background*:** Diabetes remains a global public health concern in the world. Much is known about the burden of type 2 diabetes as opposed to type 1 diabetes mellitus (T1DM) hence underdiagnosis is evident. Diabetes type 1 is often associated with multiple symptoms and patients with type 1 diabetes are left with regular insulin injection as remedy despite odds of the fact that it is sprout by multiple challenges ranging from hypoglycemia, expensive nature and inconveniences. The use of probiotic bacteria appears today as one of safer alternative to alleviate diabetes and symptoms. ***Aim of the study*:** This study aims at characterizing potential hypoglycemic probiotic lactic acid bacterium from fermented cow milk and to evaluate its effects on anthropometric parameters of type-1 diabetes mellitus (T1DM) in an alloxan-induced mice model. ***Methods*:** The lactic acid bacteria were isolated from samples of Fulani cow milk using pour plating technique on de Man Rogosa and Sharpe (MRS) agar. The isolates were then further characterized phenotypically and molecularly using the 16s rRNA gene sequencing. Type-1 diabetes mellitus was induced in Balb-c mice by administration of 150mg/Kg B.w of alloxan intraperitoneally, twice consecutively. Animals were randomly divided into 6 groups after induction with 150mg/kg of alloxan per body weight except Group I (normal control). Animals were treated with different probiotic doses of concentration: $9x10^8$ CFU/mL, $1.8x10^9$ CFU/mL and $2.7x10^9$CFU/mL respectively and insulin as positive control. MDA, NO levels as well as anti-oxidant levels (SOD, CAT, GSH) were measured from pancreatic homogenate. ***Results: One isolate (PRI 29) was selected based on its functional properties like resistance to simulated gastro-intestinal stress environment (acid and bile salt tolerance) and absence of hemolytic activity. This probiotic isolate was identified as a strain of *Lactobacillus fermentum*. The isolate was sensitive to 10 tested antibiotics including vancomycin, Imipenem and Ciprofloxacin. The isolate resisted acid and bile salts since there was no significant difference ($p > 0.05$) between viable count before and after incubation pH 2.5 and bile salts concentration of 0.3 and 0.6%. Administration of probiotic LAB significantly ($p<0.001$) ameliorated polydipsia and polyphagia. The glucose and oral glucose tolerance levels were ameliorated in probiotics treated groups $p<0.05$ as compared to the untreated group. Administration of *Lactobacillus fermentum strain* was capable of significantly ameliorating oxidative stress in LAB treated mice compared to untreated group ($p<0.001$) demonstrated by decrease in pro-oxidant NO and MDA. The administration of probiotics led to increase in anti-oxidants (SOD, GSH & CAT) to scavenge oxidative stress biomarkers within the mice treated group ($p<0.05$). ***Conclusion*:** The selected Probiotic lactic acid bacterium isolated from cow milk possess antidiabetic and anti - oxidant properties.

Keywords: Alloxan , Diabetes Mellitus type-1, Cow Milk, Lactobacillus fermentum, Probiotic Lactic acid bacteria, mice model.

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1.0 INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Diabetes type 1 emerges as a result of destruction of the beta cells of the islets of Langerhans (Noble & Erlich, 2012). The global prevalence of diabetes mellitus has observed a rise from 2019 to over 537million

representing a prevalence of over 10.5% (Saeedi *et al.,* 2019). The global prevalence of diabetes mellitus was in 2019 estimated to be 9.3% (463million people) with a forecasted rise to 10.2 % (578million) by 2030 and 10.95(700million) by 2045 (Lin *et al*., 2020). Africa is estimated to have 15.9 million adults living with diabetes mellitus representing a prevalence of 3.1% (Ogunsakin *et al*., 2021). Type 1 diabetes is amongst the least studied or priority area of research in the world with estimated overall prevalence about 5%−10% of all cases of diabetes mellitus (Mobasseri *et al.,* 2020). In Cameroon, diabetes prevalence was estimated at around 6% in 2020 (Simeni Njonnou *et al*., 2020). Diabetes type 1 is a T-cell mediated autoimmune disease in which destruction of pancreatic β-cells which results to insulin deficiency which leads to hyperglycemia and a tendency to ketoacidosis (Citro *et al.,* 2021). Diabetes type 1 is associated with microvascular and macro vascular complications. Hyperglycemia is also usually associated with polyuria, polydipsia, polyphagia and weight loss if not controlled (Jean-Marie *et al*., 2018). The management of diabetes without side effects has remained a daunting challenge to the scientific and medical community as a whole (MacCracken *et al.,* 1997; Giongo *et al*., 2011). Management of type 1 diabetes is usually by repeated administration of insulin and monitoring amelioration of symptoms in the absence of permanent curative solutions. There exists no treatment of diabetes type 1 whose control is rather dependent on regular injection of insulin to patients due to insufficiency. Pancreas transplant was forecasted as the magic bullet to resolving this dilemma however the world remains a plaque due to host immune rejection (Rakhi *et al.,* 2021). Several oral anti-diabetic drugs are widely used for associated hyperglycemic control however they have side effects. They are usually associated with leukopenia, hypoglycemia and sore throat. Insulin has notably been reported for lethal hypoglycemia and weight gain (Kalra *et al*., 2013). Metformin is a biguanide regularly used in clinical space with ability to enhance action of insulin in the liver, reduce glucose output and utilization by the muscle tissue (Caballero *et al*., 2003). Hypoglycemic agents are tiptoed to have more negative effects to the gastrointestinal tract by causing indigestion, ketonuria and lactic acidosis. With the prevailing negative effects of currently existing antidiabetic drugs, the development of new economically efficient anti-diabetic drugs/foods with minimal side effects is important. Recent studies have demonstrated a relationship between dysbiosis of gut microbiota in diabetic patients and healthy individuals which can lead to disruption of metabolism such as dyslipidaemia and insulin resistance (Varankovich *et al*., 2015; Markowiak *et al.,* 2017; X. Wang *et al*., 2021**)**. Probiotics possess hypoglycemic, anti-oxidant and anti-inflammatory activities which makes them a suitable anti-diabetic agent (Khan *et al.,* 2021**)**. Probiotics are live micro-organisms which when administered in appropriate amounts can confer potentially numerous health benefits (Markowiak & Ślizewska, 2017). Probiotic lactic acid bacteria are amongst the commonest probiotic found in fermented milk and dairy products which are generally considered non-pathogenic and do not produce toxins or harmful end-products (Axelsson *et al*, 2004; Ayivi *et al.,* 2020). Probiotics can strengthen the gut barrier functions by supporting energy for epithelial growth and resisting microbes through the production of short chain fatty acids (SCFA) (Markowiak-Kopeć & Śliżewska., 2020). These SCFA can regulate protein expression controlling satiety and hence reduce food intake amongst diabetics. Following the interesting prospects of probiotic lactic acid bacteria and their easy availability in fermented products and its suitable anti-diabetic properties, they may be the best shot for diabetes type 1. In this regards the objective of this research was to isolate, characterize probiotic lactic acid bacteria from fermented cow milk and evaluate its effects on hyperglycemia and anthropometric parameters in an alloxan induced diabetes mellitus mice model.

2.0 MATERIALS AND METHODS

2.1. Sample collection and Media preparation

The samples used in this study was Fulani fermented cow milk collected from the Far North Region of Cameroon. Samples collected from Cattle rearers under aseptic conditions. Spot sample collection was adopted with respect to acquisition of milk samples. These samples were placed in a wide mouth container of 500ml with a lid to avoid contamination. The samples were transported to the University of Buea for continuous preservation till commencement date of experiments in ice blocks with aid of a transport flask. Nine (9) mL of normal saline (0.85%) was pipetted into test tubes and autoclaved. After autoclaving, it was allowed to cool and a ten (10) fold serial dilution was performed under aseptic conditions to avoid contamination. The media preparation was done based on manufacturer's instruction stipulating the suspension of 68.3g MRS agar in 1litre of distilled water. The samples were analyzed in the south west region precisely Buea in Fako division. The research was carried out in the University of Buea involving multiple collaborations: Faculty of Science teaching laboratory, Biotechnology laboratory.

2.2. Isolation and molecular identification of lactic acid bacteria from Fulani fermented milk by the sequencing of the16 S rRNA gene.

2.2.3 Isolation of Lactic acid bacteria

One (1) mL aliquot of three different dilution (10^{-5}, 10^{-6} and 10^{-7}) of the sample were poured into plates and about 15mL of De man, Rogosa and sharpe (MRS) agar (Sigma-Aldrich, Germany) was added under aseptic conditions beside a flame and allowed to solidify at room temperature and the plates were sealed using masking tape. The plates were then incubated

upside down at 37◦C to avoid contaminations for 24-48h. The isolated colonies were subjected to preliminary screening using catalase test. Screening and identification of selected Lactic acid isolates was conducted by phenotypic and morphological characterization (shape, colour, elevation etc). Isolates that demonstrated catalase negative characteristics were selected and sub-cultured on MRS agar to confirm distinct growth. The presumptive isolates were subjected to catalase and gram staining and catalase negative isolates were retained as presumptive of lactic acid bacteria. The isolates were kept at 4 ◦C in 1.5 mL sterile Eppendorf tubes containing MRS broth for further investigation (physiological, biochemical and functional characterization etc) in 20% glycerol.

2.2.4 Motility test, growth at varying temperature

Motility was performed as described by (Cousin *et al*., 2015) with slight modifications; briefly by stabbing the agar and checking for diffusion. MRS broth culture of presumed lactic acid bacteria within 24h and 10mL of prepared MRS agar was poured into tubes and allowed to solidify with inoculation of LAB. Growth temperature was performed as described by *(Cousin et al.*, 2015), briefly a colony was inoculated into MRS broth media and incubated at temperatures 30ºC and 37ºC for 24-48h and the bacteria growth was observed by the formation of sediment in the media.

2.3. Functional characterization and safety evaluation

For probiotics to be efficient they should be able to resist gastrointestinal stress environment as such they should be able to resist low pH and bile salts in the small intestines.

2.3.1 Acid tolerance and Bile Tolerance

The acid tolerance test in this study was done as described by Tatsinkou *et al*., (2017). Briefly overnight culture of the isolate in MRS broth was centrifuged at 4000rpm at 4ºC. Pellets were recovered and washed 3times, after which plate counting approach was used to enumerate the cell in the pellet at 0h. About 1mL of pellet suspension was incubated in MRS broth with adj usted pH (2.0, 2.5, 3 and 3.5) for 3h. After 3h, 1mL of each culture was serially diluted and plated on MRS agar to enumerate the bacterial cells. MRS broth was autoclaved and when ready transferred 1ml of broth with acid into Eppendorf tubes. Each isolate was then inoculated into Eppendorf tubes in triplicates while ensuring proper labelling and incubated for 3hours. The isolates were then later removed from the incubator and washed 3times in PBS and serial dilutions of sediments done. Plate count was done to count the microbial growth alongside a normal control.

The method used for testing bile tolerance used was similar to that described by (Argyri *et al*., 2013). The bile used in our study was oxgall which is a natural dried bovine bile component often used for this test. LAB isolates were cultured in MRS broth, for 24 hours at 37°C. 100 µL of each cell suspension were inoculated into bottles containing 10 ml MRS broth to which 0.3 % (w/v) oxgall-bile, 0.60 % (w/v) oxgall-bile and one set of MRS broth without oxgall-bile to serve as the control. The cultures were incubated simultaneously at 37 °C and counted from the pour plate. Growth reflected by increase in the number of isolates was considered as been tolerant to lactic acid bacteria isolates to bile.

2.4 Safety evaluation of isolated lactic acid bacteria

2.4.1 Antibiotic sensitivity testing of Isolates

Antibiogram test was performed to record the sensitivity or resistance of LAB towards conventional antibiotics. The standard disc diffusion assay was employed to analyze antibiotic sensitivity pattern through of the selected isolate according to the method described by (Bauer *et al*., 1966) with slight modification. In brief, broth culture (100ul, 0.5 McFaland equivalent to 10^8 CFU/mL) of all the tested strains was mixed in 8mL of soft agar, allowed to solidify and antibiotic discs were aseptically placed equidistant to each other using Sterile Forceps. The culture plate was pre-incubated at room temperature for 1h to ensure proper diffusion and then incubated overnight at 37°C. After overnight incubation, the diameter(mm) of zone of inhibition (ZOI) and results were depicted as resistant(R), intermediate(I) or sensitive(S). According to clinical laboratory standards institute CLSI (2021) interpretive category, S signifies that tested isolates are inhibited, R implies isolates are resistant, isolates with less than 14mm were considered resistant, those with 20mm as sensitive and those between 15-19mm as intermediate.

2.4.2. Hemolytic Activity

The hemolytic activity of isolates was determined as described by (Somashekaraiah *et al*., 2019a) briefly using Columbia agar containing 5% (w/v) sheep blood, plates were incubated at 37 °C for 48 h. After incubation, the hemolytic activity of isolated strains was evaluated and classified on the basis of lysis of red blood cells in the medium around the colonies. For hemolytic activity, the overnight grown MRS broth culture of the lactobacilli strains was streaked on blood agar plate (Hi-Media, India) and incubated at 37 ◦C for 72 h; thereafter, the plates were observed for the formation of any clean (β-hemolysis) or greenish (α-hemolysis) hemolytic zones, or no such zone (γ-hemolysis) around the lactobacillus colonies.

2.5. Molecular identification of probiotic bacterium

PCR amplifications was done by using universal primer for 16S rDNA under following conditions: 5 min at 95 ºC, 30 cycles of 40 s at 95 ºC, 60 s at 53ºC, 2 min at 72 C and final extension at 72 C for 5 min. Amplified PCR product of LAB isolates

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were sequenced at INQABA Genomics, South Africa. Nucleotide sequences were analyzed for similarities in NCBI by BLAST and related sequences were obtained from NCBI. Sequencing was done using universal primer (F)5'AGAGTTTGATCCTGGCTCAG 3' ,5'ACGGCTACCTTGTTAACGACTT 3'(R). The 16S rDNA sequences were analyzed using the GenBank database, and identification was performed on the basis of 16S rDNA sequence homology on NCBI.

2.6. Preparation of probiotic inoculum

Preparation of probiotics inoculum was done in order to administer to animals at different concentrations in the experimental groups. MRS agar was prepared following manufacturer's instructions and cultured to check for viability and read after 18-24hrs. Once there was growth, the isolates were morphologically characterized and their catalase activity determined. MRS broth was then prepared and 1ml transferred into 1.5ml Eppendorf tubes. Bacteria cells for *in-vivo* studies were grown on MRS broth overnight at 37 ◦C, then separated from the culture supernatant by centrifugation (4 min at 4000×g) at 4 ◦C, washed three times with ice cold phosphate buffer saline (PBS) (pH = 7.2) and re-suspended in PBS. The isolated probiotic lactic acid bacteria was adjusted to 3 concentrations thus; McFarland standard 3, 6 and 9 corresponding respectively to 9×10^8 cfu/mL, 6 (1.8×10^9 cfu/mL and 2.7×10^9 cfu/mL using spectrophotometry.

2.7. Experimental animals and ethical consideration

Young male Balb-c mice (6-8 weeks old) and average body weight (between 22-29g) were used for the experiments. They were bred in the Animal House of the Faculty of Science, University of Buea, maintained under standard conditions (clean cages placed in a well-ventilated house condition with a temperature of 22 ± 2 °C and photoperiod of 12 h light and 12 h dark cycle). All mice used were Balb-c mice from same supplier to avoid variations (UCL, 2021; GCULA, 2011). All animals were housed and kept in cages group in an approved space by the IACUC. All experiment was performed was based on recommendations from international association for animal ethics (IACUC).

2.6.2. Induction of diabetes in experimental animals

Diabetes was induced in overnight fasted male Balb-c mice by a 2 dosage of alloxan intraperitoneal injection (i.p) of freshly prepared solution of alloxan (150 mg/kg body weight) in 0.1 M citrate buffer (pH 4.5). The animals were confirmed diabetic by the elevated plasma glucose levels after 72 h of injection. The mice with stable hyperglycemia (blood glucose >8.1 mmol/L) were randomly divided into the experimental groups.

2.6.3. Animal grouping and probiotic dosage

Mice were randomly divided into six groups of six mice each per cage. Thirty-six male mice were randomized into six groups consisting of six animals in each group. Group I: normal control mice administered with PBS daily for 14 days; Group II: diabetic animals received PBS ; Group III-V : diabetic mice, were treated daily with probiotics McFarland standard 3 (9×10^8 cfu/mL), 6 (1.8×10^9 cfu/mL) and 9 (2.7×10^9 cfu/mL) respectively; Group VI: diabetic animals received 0.075IU of insulin only. All animals from each group were sacrificed after their respective daily dosages of the extract and distilled water.

2.6.4. Measurement of blood glucose, oral glucose tolerance and anthropometric parameters

About 50-75µL of blood was collected from the tail of each mouse and postprandial blood glucose level was measured using fine care glucometer (Auto-coding Premium, Obellis S.A). Oral glucose tolerance (OGTT) was performed 2 weeks after Lactobacillus fermentum strain treatment. Mice were fasted for 12 h and blood glucose levels (0 min) were measured. Thereafter, mice were orally administered glucose (2 g per kg body weight), and their blood glucose levels were measured at 30, 60mins, and 120 min using fine Test glucometer (Auto-coding Premium, Obellis S.A). Body weight, water intake and food intake were taken periodically for 2weeks.

2.7. Measurement of Oxidative stress parameters

2.7.1. Determination of oxidative stress biomarkers (MDA and NO).

Pancreatic Nitric oxide level was measured using the Greiss reagent as described by Taiwe *et al*.,(2015) and. Briefly, 0.5 mL of Greiss reagent and 0.5 mL of pancreatic supernatant were introduced into a test tube and allowed and absorbed read 5 minutes, and absorbance read at 540 nm using a spectrophotometer (Thermo Scientific; MultiskanTM FC Microplate Photometer). A standard curve(sodium nitrate) was used to express the concentration of NO in mol/g. Pancreatic MDA peroxidation level was measured using the thiobarbituric assay and optical density of the supernatant was read using a spectrophotometer (Thermo Scientific; MultiskanTM FC Microplate Photometer) at 546 nm. The pancreatic MDA levels was calculated in µmol/g using Beer Lambert's formula and the extinction coefficient of:1.6 ×105 M/cm (Yuwong Wanyu *et al*., 2022).

2.7.2 Determination of anti-oxidant biomarkers (GSH, CAT and SOD)

Pancreatic reduced glutathione (GSH) was determined using Ellman's reagent as originally described by Ellman in the manuscript by (Aluwong *et al*., 2016) and Ghani *et al*.,2017 with little modifications. A volume of 1.5 mL of Ellman's reagent was added to 100 µL of pancreatic sample and kept at

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room temperature for 1h, and absorbance read at 405 nm using a spectrophotometer (Thermo Scientific; MultiskanTM FC Microplate Photometer).

To measure catalase levels this was done as described by (Childs *et al*., 2017). Briefly, equal volumes of 125 µL of supernatant and of 0.1 M phosphate buffer (pH 7.4) and 0.5 mL of 30 mM of hydrogen peroxide (H2O2) were mixed and absorbance was read at 240 nm for 30 s, 60 s, and 90 s using a spectrophotometer (Thermo Scientific; MultiskanTM FCMicroplate Photometer). Catalase activity was expressed as mmol of g of tissue.

2.8. Statistical analysis

Statistical analysis was performed using Microsoft Excel, Graph Prism 8.4.3 and all data were presented as mean ± standard deviation (SD). Statistical difference between groups were conducted using one-way ANOVA followed by a Turkey test. A statistical significance was set at 95% confidence interval (p-value inferior to 0.05).

3.0 RESULTS

3.1.1 Isolation and characterization of Probiotic lactic acid bacteria (LAB)

Upon isolation, isolate PRI 29 was a small opaque colony, circular in shape with cream colour and entire margins. The isolate also revealed a catalase negative test as well gram-positive test. The isolate did not present hemolytic activities and was sensitive to all antibiotics tested. It was also non-motile and grew at temperatures between 30° and 37°C. Table 1 summaries the results of cultural, morphological and biochemical characteristics and the antibiogram of the isolate PRI 29.

Table 1: Characterization of *Lactobacillus fermentum* (PRI29) isolated from fermented milk

Cultural Characteristics		Morphological/Biological Characteristics			Antibiogram	Antibiotics sensitivity testing **Antibiotics (N=10)**			
Variable	description	Variable	description			Antibiotics disc	Sensitivity	Antibiotics disc	Sensitivity
Colour	Cream	Gram Stain	Positive			Va(30µg)	S	E (30µg)	S
Shape	Circular	Catalase	Negative			Imi(10µg)	S	Amox(30µg)	S
Elevation	Raised	Shape	Short rods			Ctx(30µg)	S	Do (30µg)	S
Type	Small	Hemolysis	No hemolysis			CN(5µg)	S	CRO (5µg)	S
Opacity	Opaque	Motility	Non-motile			CIP (5µg)	S		
Margin	Entire	Temperature	30	+		TE(30µg)	S		
			37	+					

+ = positive, Va = vancomycin, Imi=Imipenem, CIP = Ciprofloxacin, Amox = Amoxicillin, Do = doxycycline, CRO = Ceftriaxone, CN= Gentamicin, Tet = Tetracycline, Ctx = cefotaxime, E = erythromycin, TE = tetracycline, S=sensitive

3.1.2 Physiological properties of *Lactobacillus fermentum strain (PRI* 29) isolated from fermented milk

Isolate PRI 29 resisted acid and bile salt conditions since there was no significant difference (at p>0.05) in their viable count before and after incubation at pH 2, 2.5, 3 and 3.5 for 3h and at 0.3% ,0.4% and 0.6 % bile salts for 4H. Isolate PRI 29 had a survival rate of 94.23% and 95.85% at selective resistance pH of 2.0 and 3.0 and there was no significant difference from the control value (p>0.05). Bile concentration fluctuates between 0,2-0.3% bile concentration hence beneficial lactic acid bacteria must be able to resist bile salt at stipulated concentrated and beyond. Isolate PRI 29 resisted bile concentration at 0.3% (94.18 % survival rate) and 93.46% survival rate at o.4% bile presenting no statistical difference (p> 0.05).

Table 2: Acid and bile tolerance of *Lactobacillus fermentum* (PRI 29)

Acid tolerance			Bile Tolerance		
Variable	**Viable count (LogCFU/mL)**	**Percentage survival(CFU/ml)**	**Bile Concentration**	**Viable count (Log CFU/mL)**	**Percentage survival**
pH 7.2	8.68 ±0.16		**0h**	8.42±0.08	
pH 2.0	8.18±0.22	94.23%	**0.3%**	7.93± 0.11	94.18%
pH 2.5	8.22±0.13	94.70%	**0.4%**	7.87±0.18	93.46%
pH 3.0	8.32±0.28	95.85%	**0.6%**	7.21±0.6	85.62%
pH 3.5	8.36±0.17	96.31%			

0h = Control, pH 7.2 = Control pH

3.1.3 Molecular identification of isolate PRI 29

Isolate PRI 29 which had presented good acid and bile tolerance coupled with their preliminary identification were subjected to DNA extraction followed by PCR assay using the 16s rRNA universal primers in order to confirm the identity of bacterium.

The results obtained showed PCR amplification product between 1.5-2.0 k.bp. The isolate PRI 29 was identified after blasting as *Lactobacillus fermentum* strain with a percentage identification of 99.05% and associated accession number of NR 113335.1. The isolate had an ascension length of 1501.

3.2. Variation of Body weight, food intake and Water on administration of probiotic *Lactobacillus fermentum* in alloxan induced type 1 mice model

In our study during week 1 there was no significant difference (p>0.05) observed in body weight between negative control group and low to high dose treatment group. However, an increase in body weight was observed in the insulin treated group at week 1 and 2 (p<0.001). In our study compared to the untreated group, Body weight increased in all treated groups except low dose which barely maintained a fairly constant weight and untreated group which observed a decline in body weight (see Table 3). The water intake increased increased in untreated mice from 104±3.12 in week 1 to 135±4.26 at end of week 2 compared to normal control (p<0.001).

There was a significant decrease in food intake (p >0.001) from 132.0±3.02 g in the normal control group to 104.0±3.12 in the untreated group (negative control) at week 1. There was a significant increase in food intake from 132.0±3.02 g in normal control group to 172±1.72g in the untreated diabetic group (p>0.001).

Table 3: Effect of *Lactobacillus fermentum* on body weight, Food and Water Intake in alloxan induce mice model

		Group I	Group II	Group III	Group IV	Group V	Group VI
Body weight (g)	Week 1	26.62±1.45	25.20±1.15	27.21±0.00	26.20±2.08	27.40±1.00	25.27±1.09
	Week 2	27.73±1.76	20.54±1.62###	22.72±0.00	28.33±1.76	29.00±0.57	29.86±1.97***
Food intake(g)	week 1	132.00± 3.02	104±3.12###	153.50±3.94 ***	144.60±6.02**	128.40±1.85	145.80±3.03
	week 2	135.00± 2.10	172±1.72 ###	127.00±2.18	122.00±2.54	112.00±2.16	137.00±1.30
Water intake(ml)	week 1	95.00±10.41	103±5.51	110.00±4.41	132.00±4.93**	205.00±10.88***	118.00±2.58*
	week 2	107.00±2.68	135±4.26###	120.00±2.04**	112.00±2.39	100.00±8.66***	103.00±9.06***

Data were analyzed using the one-way ANOVA and expressed as mean±SEM (n=6). *p<0.05, ***p<0.001, **p <0.01 Vs Group II. Group I: normal control; Group III: Probiotics MCF 3, Group IV: Probiotic McF 6 , Group V: Probiotic McF 9, Group VI : Positive control insulin.

3.3 Effects of Lactobacillus fermentum on Glucose Level and Oral Glucose tolerance

3.3.1 Variation of Glucose on administration of Glucose week 1 and 2

Figure 1 below shows the variation of blood glucose level over the weeks upon administration of probiotics *Lactobacillus fermentum strain* to mice induced with diabetes type 1. The glycemia level in untreated diabetic (Group II) mice observed a significant continuous increased glycemia levels over 2weeks *(p<0.001)* when compared to normal group (Group I). Diabetic treated groups observed a significant decrease in blood glucose levels after 2weeks *(p<0.001)* when compared to the negative control (Group II).

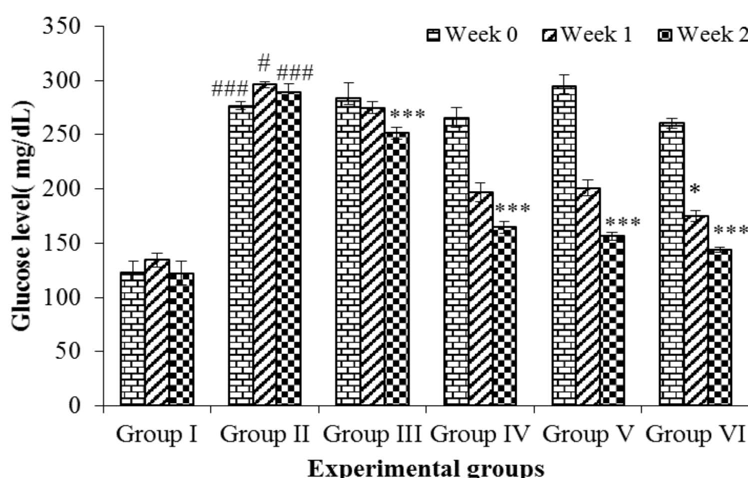

Figure 1: Illustration of variation Glucose on administration of probiotic *Lactobacillus fermentum strain* to type 1 diabetes mellitus mice

Data were analysed using the one-way ANOVA and expressed mean±SEM (n=6). ###p<0.001, vs Group I; *p<0.05, **p<0.01 and ***p<0.001 vs. Group II. Group I: normal control; Group VI: Positive control insulin; Group I: Negative control, Group III: Probiotics (*Lactobacillus fermentum* strain) McF3, Group IV: probiotics (*Lactobacillus fermentum*) MacFarland 6, Group V: probiotics (*Lactobacillus fermentum strain*) McFarland 9.

3.3.2 Effects of *Lactobacillus fermentum strain* on 2hrs Oral Glucose tolerance Test

The oral glucose tolerance test is a diagnostic parameter important to verify the hypoglycemic effects of *probiotic Lactobacillus fermentum strain* NBRC15885. There was a significant higher level of glucose in untreated diabetic mice (Group II) when compared to the normal group (Group I) with p<0.001 at end of 2h glucose load. The treated groups observed significant decrease in glucose levels with administration at lower dose leaving the animal still time as seen in Table 5. The was a significant difference between untreated group (Group II) and positive control group.

Table 4: Effects of Probiotic potential of *Lactobacillus fermentum strain*

Time	Group I	Group II	Group III	Group IV	Group V	Group VI
0	122.33±11.20	289.67±7.88$^{\#\#}$	251.33±5.21*	154.67±5.48	136.33±3.48**	144±2.08***
30	253±19.31	335.67±3.53$^{\#\#\#}$	336.67±16.69*	245±14.57**	281.33±17.02**	284.33±13.37**
60	194±15.89	348±2.33$^{\#\#\#}$	313.33±7.22**	221±7.51	254.67±20.20***	191±3.21***
120	132.33±6.90	356±5.86$^{\#\#\#}$	274.73±3.53***	160±2.65	142.33±12.55***	104±3.51***

Data were analysed using the one-way ANOVA and expressed mean±SEM (n=6). $^{\#\#\#}$p<0.001, vs Group I; *p<0.05, **p<0.01 and ***p<0.001 vs. Group II. Group I: normal control; Group VI: Positive control insulin; Group I: Negative control, Group III: Probiotics (*Lactobacillus fermentum strain*) McF3, Group IV: probiotics (*Lactobacillus fermentum strain*) MacFarland 6, Group V: probiotics (*Lactobacillus fermentum strain*) McFarland 9.

3.4 Effects of administration of *Lactobacillus fermentum strain* on Oxidative stress.

3.4.1 Effects of *Lactobacillus fermentum strain* on oxidative stress markers.

The induction of diabetes type-1 using alloxan resulted in a significant increase in MDA levels from 14.43±0.32 in the normal control (Group I) to 27.89±1.22 in the untreated group (Group II) with p<0.001. There was a significant decrease in the MDA oxidant levels in the treatment groups with increasing concentration with the most significant decrease observed at McFarland's 9 which observed a decrease from 27.89±1.22 in the untreated group to 14.81±0.03 with p<0.001. The positive control group (Group VI) administered insulin equally observed a decrease in MDA values to 14.08±0.03 compared to the untreated group (p<0.001) as shown in figure A. Pertaining to Nitric Oxide, there was a significant increase in NO levels from 1.961±0.18 in Group I (normal control group) to 3.95±0.38 in Group II (Negative control) with p<0.001). There was a significant decrease in NO levels from 3.95±0.38 at probiotic administration representing McFarlands 6 (p< 0.05) to 2.74±0.24 compared to Group II (negative control group) and 1.82±0.19 at McFarland 9 (p<0.01) as seen in Figure 2(B).

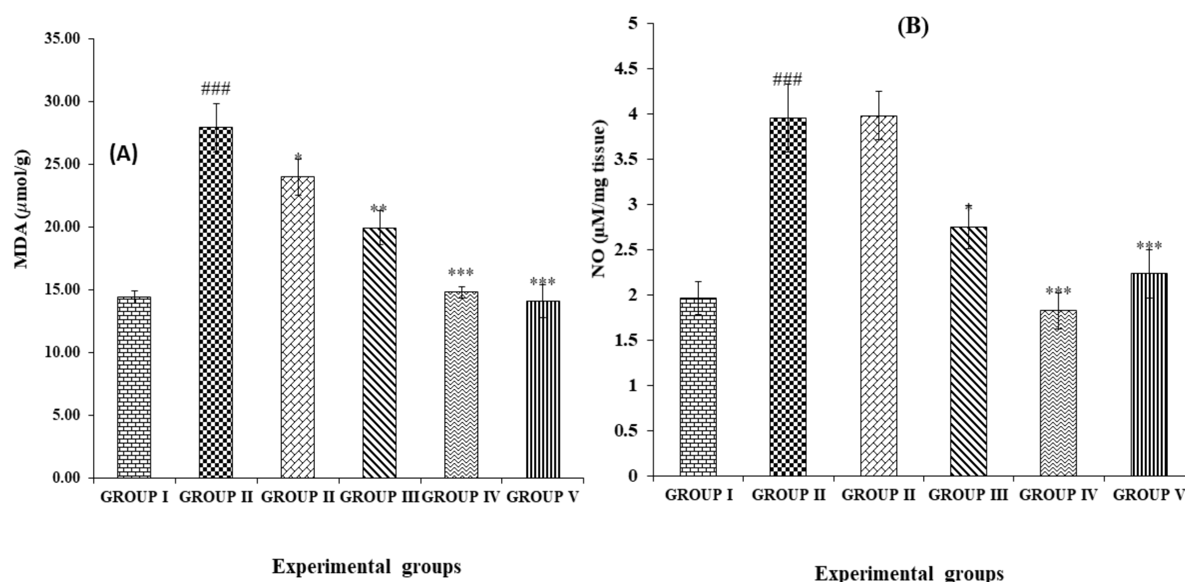

Figure 2: Illustration of effects of *lactobacillus fermentum* strain on oxidative stress biomarkers

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3.4.2 Effects *of Lactobacillus fermentum strain* anti-oxidant activity

The anti-oxidant effects of *Lactobacillus fermentum* was evaluated in order to investigate the ability to scavenge reactive oxygen species such as Catalase (CAT), Superoxide dismutase (SOD) and Glutathione (GSH). Superoxide dismutase observed a significant reduction in Group II (untreated group) after induction of diabetes by alloxan from 25.59±2.27 in Group I (normal control) to 13.21±0.81 (p<0.001). The SOD levels significantly increased with probiotic *Lactobacillus fermentum dosage* from 13.21±0.81 (untreated group) to 20.18±1.27 at McFarland 9 (p<0.001). Catalase levels decreased significantly reduced in Group II (untreated group) from 0.53±0.04 in the normal group to 0.28±0.03 (p<0.01). Catalase levels were increased in Group V (Probiotic treated group McFarland 9) from 0.28±0.03 in the untreated group to 0.47±0,03 (p<0.01). Levels of GSH were significantly decreased from 4.40±0.41 in Group I (normal group) to 1.76±0.25 in group II (untreated) with p<0.001. GSH observed significant increase levels in Group V and VI when compared to group II to 4.16±0.26 and 4.60±0.19 respectively (see Fig D).

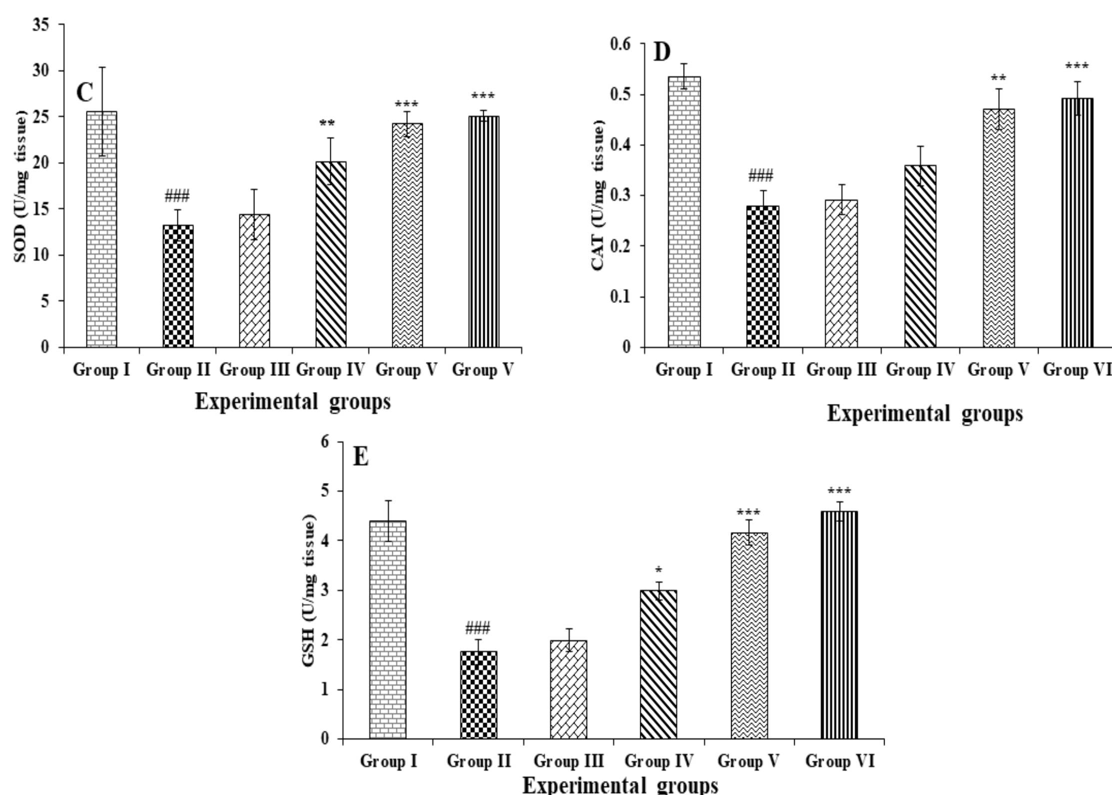

Figure 3: Illustration of effects of *lactobacillus fermentum* strain on anti-oxidant stress biomarkers

3.6 DISCUSSION

Cow milk is amongst highly consumed products in low- and middle-income countries amongst cattle rearers including Cameroon. The dynamics of time has however made the consumption of these products in disproportionate ratios amidst potential health benefits. Identification of lactic acid bacteria strains from these fermented products to characterize and assess their safety properties is paramount to fully exploit their health benefits. Probiotics has gained increasing attention over the years to the food industry and health. Lactic acid bacteria are most common member of this family used for fermentation and preservation of food etc (Fijan, 2014; Marco *et al*., 2021). In the western world probiotic supplements exist, however in low- and middle-income countries or sub-Saharan Africa resorting to fermented milk products is a way out to get same health benefits Lamprecht *et al*., (2012). There exist several traditional fermented products whose probiotics potentials aren't well exploited (Somashekaraiah *et al*., 2019b). The isolate PRI 29 (*Lactobacillus fermentum strain*) from unpasteurized milk were isolated on MRS agar with cultural characterisation ranging from (cream, Circular entire, raised or flat, small and opaque). The above descriptions from our study closely highlighted features of presumptive lactic acid bacteria and similar to results obtained in Cameroon by (Fossi *et al*., 2015) *who obtained lactic acid bacteria from palm-wine with similar characteristics. Similar results were obtained* in Turkey where they isolated probiotic *Lactobacillus fermentum, Enterococcus faecalis, Enterococcus*

durans, and Lactobacillus helveticus species. L. fermentum and L. helveticus from fermented koumis and Kurut (Ispirli & Dertli, 2018). The isolate was gram positive with rod shape and catalase negative. This was similar to results obtained in a study carried out in India on sludge dairy products as well as Cameroon (Shabana *et al.,* 2013; Toukam *et al.,* 2021). The presence of probiotic lactic acid bacteria in milk could be explained by the fact that Lactic Acid Bacteria (LAB) produce lactic acid that is capable of growth at lower pH values than other bacteria (pH 4.0 – 5.0) reason for which it is used to ferment milk to make products such as yoghurt etc. This isolate was also non-motile from the test of motility, this was in agreement with a study which stipulated lactic acid bacteria isolated from local cow´ milk kefir was non-motile (Ismail *et al*., 2018**)**. These results could be explained by the fact that probiotic lactic acid bacteria lack flagella and hence couldn't have produced colonies that spread over agar. Probiotics despite enormous health benefits must be screened for safety. Screening for safety was performed by using hemolysis and antibiotics resistance**.** Safety properties are important to evaluate because to ensure lack of hemolytic activity during the selection of probiotic strains, because such strains are non-virulence and the lack of hemolysin ensures that virulence will not appear among the bacterial strains (FAO/WHO 2006)(Monika *et al.,* 2017). The isolate PRI 29 showed gamma hemolysis. Our findings were similar to those obtained by screening 10 probiotic Lactic acid bacteria from non-dairy foods by reported by (J. Wang *et al*., 2018). The main concern regarding the safety of probiotics is their antibiotic resistances since these strains may transfer antibiotic resistance genes to pathogenic bacteria, which may represent a serious risk for the treatment of infections (Ayivi *et al*., 2020; J. Wang *et al*., 2018). Isolate PRI 29 was found sensitive to all antibiotics used as shown on Table 1.

It is worth reiterating on the fact that the evaluation of the functional and safety properties of probiotic strains in-vitro which includes transit tolerance in stimulated upper gastrointestinal tract(Hassanzadazar *et al*., 2012). Bile salt hydrolase activities are considered important factors for selecting novel probiotics strains (Feng *et al*., 2017a). The microorganisms should cross over several biological barriers through the GIT, including gastric acid, enzymes, secretions and bile salts. Hassanzadazar *et al*., (2012) reported that hydrochloric acid found in human stomach disr upts biomolecules of cells, such as proteins, genetic material and fatty acids. Acidic pH can inhibit reduce metabolic rate and diminish the growth and viability of Lactobacilli. Isolate PRI 29 *(Lactobacillus fermentum strain*) showed resistance at pH 2.0 and isolate with mean count greater than 6log cfu/L and showed a slow increase in the viability at pH 2.5 (Table 2). *Lactobacillus fermentum strain* observed an increase in colony count from pH 3.0-3.5 however there was no significant difference when compare to the normal contro**l.** These results are similar to those obtained by (Maldonado & Nader-Macías, 2015a) who demonstrated the variability of resistance amongst lactobacilli species isolated from young cow's milk to maintain fairly stable counts. This could be explained by the fact lactobacillus species have adaptive abilities to acid at the time of their presence in MRS agar or broth which indicates potential survival in GIT. Previously researchers had established threshold for resistance to acid to be at pH=2 and pH=3 for 3hrs incubation, as it is believed to be the time required to stimulate bacteria residency in the stomach hence PRI 29 fulfilled the conditions (Maldonado & Nader-Macias, 2015b; Prasad *et al*., 1998**)**.

Bile tolerance was evaluated at concentrations of 0.3 and 0.6% to assess resistance to stomach bile. Isolates PRI 29 representing *Lactobacillus fermentum* strain in our study demonstrated resistance to bile salt at both 0.3% and 0.6 % concentration however isolates PRI 29 had slight decrease in the viable count at 0.6% than 0.3% however not statistically significant with p-value >0.05. This study was similar to that reported by (Feng *et al*., 2017b). Our results where different from that reported by (Ispirli & Dertli, 2018) who noted that screening of traditional koumis and kurut had variations in species survival (with possible drop of upto 24-48% at higher bile tolerance concentration). This difference could be as a result of the fact that *Lactobacillus fermentum* species process EPS due to possession of levansucrase gene responsible for production of fructan EPS. There might be also differences in gene mutation and/or cell surface characteristics based on sample sources. The combined tolerance effects of acid and bile maybe due to capsular structure of EPS protects probiotics from harsh gastrointestinal conditions like gastric juice, bile salt and e.t.c (Abukhader *et al.,* 2022; Tilahun *et al*., 2018).

Diabetes mellitus type 1 remains underdiagnosed and under-represented in present global context. Patients are sadly exposed to lifelong insulin intake which has several inconveniences ranging from overweight, hypoglycemic effects and scarcity. There is urgent need to explore and assess new natural avenues for antidiabetics with less negative effects. It is for this reason that probiotics as reported by researcher to have antidiabetic properties needs to be examined. In our study during week 1 there was no significant difference observed in body weight between untreated group and low to high dose treatment group. However, an increase in body weight was observed in the insulin treated group at week 1 and 2. In our study compared to the untreated group, body weight increased in all treated groups except low dose which barely maintained a fairly constant weight and untreated group which observed a decline in body weight (P<0.05). The results of our study were partially in line with study by Taye *et*

al., (2020) highlighting that administration of T. schimperi extract prevented significant weight loss in the experimental group. Our results slightly weren't consistent with that of Acharya *et al.,* 2012 who witnessed a rapid decline in body weight after the 3rd week in the vehicle group, this maybe as a result of the fact that our study was done in 2 weeks. Czech *et al*., (2017) stipulated that weight loss in diabetic patients is due to insulin deficiency which leads to reduction in amino acids uptake by tissues and due to declined protein synthesis resulting in lipolysis. The capability of probiotic extracts to defend weight loss may be due to its ability to scavenge free radicals or control muscle loss as well as protect the islets from devastating damage. Polyphagia and polydipsia represent classical signs of diabetes type 1 (Snell-Bergeon & Dabelea, 2009; Graham *et al*., 2012). The results obtained in our study showed increase in food in-take and water consumption in alloxan induced mice model compared to the normal control group. The increase in food consumption could be as a result of decrease in activity of leptin at the hypothalamus following insulin deficiency or a reduction in the release of some hormones promoting satiety cholecystokinin, peptide YY and glucagon-like peptide-1. Our study demonstrated increase in water consumption ($p<0.001$) in the untreated group (Group II). Our results were similar to those obtained by (Oyedemi *et al*., 2011). This increase could be explained by the fact that the marked hyperglycemia leads to exclusion of urine volume with loss of glucose. However probiotic administration of *Lactobacillus fermentum strain (PRI 29)* administered at dose representing Mcfarland 3, 6 and 9 ameliorated polyphagia and polydipsia. Diabetes is characterized by hyperglycemia and responsible for multiple organ complications (Ci, 1996; Forouhi & Wareham, 2019). Hyperglycemic control in patients with type 1 diabetes is usually through repeated administration of insulin which has been associated with possible lethal hypoglycemia. Our study demonstrated all treatment groups observed reductions in glycemia levels with highest at dosage of McFarland 9. There was a significant difference in glycemia level in the diabetic group compared to the treated groups $p<0.01$. Oxidative stress is considered to be the major factor contributing to development of tissue injury and diabetic complications. Oxidative stress represents a critical assessment avenue for detecting the healthiness of beta cells of the islet of Langerhans (Leenders *et al*., 2021). Our study demonstrates that induction of diabetes by alloxan is associated with oxidative stress as was demonstrated by a significant increase in MDA and NO levels. This could be explained by the fact that presence of hyperglycemia induces production of reactive oxygen species (ROS). However, the administration of probiotics *Lactobacillus fermentum strain* at higher doses reverted the high levels of MDA and NO significantly. This was consistent with results reported by Aluwong *et al*., (2016) who reported higher MDA concentration indices in untreated rats and demonstrated that administration of probiotics and Vit C ameliorated the status. Our results are also in line with those reported by Zhang *et al*., (2018) who reported the use of *Zanthoxylum bungeanum* extract ameliorated a high nitric oxide concentration in diabetic mice. This study reported low levels of anti-oxidants (GSH, CAT and SOD) in the diabetic grouped(group II) however significant improvements in levels of anti-oxidants were observed with the administration of Probiotic *Lactobacillus fermentum*($p <0.001$). This could be explained by the fact that administration of probiotics impaired lipid peroxidation pathways. In the present study, low activities of antioxidant enzymes in the Group II (untreated diabetic mice) were recorded. This finding further supports previous results that diabetes is associated with impaired antioxidant defences (Aluwong *et al*., 2016).

CONCLUSION

The results of this research shows that probiotic lactic acid bacteria are found in local fermented milk and possess potential antidiabetic properties as well as ameliorating the symptoms that arises from diabetes type 1. Lactobacillus fermentum strain possess antidiabetic properties. *Lactobacillus fermentum* strain from Fulani cow milk has ability to reduce oxidative stress by production of anti-oxidant levels to act as scavengers for the generated reactive oxygen species.

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Data Availability

The data for this article are available upon request from the corresponding author and 1st author stated forth (tatsinkou.fossi@ubuea.cm, bobgatanyi@yahoo.com).

Conflicts of Interest

The authors declare that they have no conflicts of interest. Authors'

Contributions

BTF, LMN and PTB conceived the study: TBF, SGT and PTB implemented the study. TBF and LMN supervised the study. NK, NEY, WBY, NFA and PBT participated in laboratory and data analysis. TPB drafted the first manuscript; TBF, LMN, SGT and LLTT reviewed and corrected the manuscript. All authors approved the final copy.

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