Original Research Article

Identification of Probiotic Lactic acid bacteria from Palm wine and their in-vivo evaluation on Diabetic patient using wistar rat

Abstract

Palm wine consumption has been used as a means of merriment in ceremonies and festive periods, however can diabetic patient consume it? This present study hopes to find an answer to this question and exam the microbial contribution to this effect. In this present study, bacteria inhabiting the two palm wine sources were identified using the gold standard for cultivation, characterization and identification (molecular analysis using Polymerase Chain Reaction (PCR) techniques and Deoxyribonucleotide (DNA) sequencing). Only one isolate was obtained known as *Weissellaconfusa*. The isolate was subjected to probiotic selection and was found fit for consumption and would be beneficial if consumed by diabetic patient. Based on the outcome of this study, diabetic patient are advised to consume palm wine.

Introduction

Palm wine is a type of a whitish effervescent alcoholic beverage produced from sap of various palms through the process of fermentation. Examples of palm sap in which palm wine are extracted from are*Elias guineensis, Raphia regalis, Raphia sudanica, Raphina.vinifera* and *Raphinahookeri* (Obire, 2005). Palm sap has some known microorganism such as *Saccharomyces cerevisiae*. The fermentation of palm wine involves a group of bacteria known as lactic acid bacteria (*Leuconostoc, Pediococcus, Micrococcus, Acetobacter* and *Lactobacillius* which can undergo heterofermentative or homofermentative fermentation), yeast (*Sacchromycescerevisae, Candida* spp.), acetic acid bacteria and probiotics (Zymomonas and Leuconostoc are normal flora of a palm sap). The type of microorganism present depends on the stage of the fermentation and the composition of the palm sap at that point (Akinrotoye, 2014).

Statement of Problems

Palm wine has several relevance such as the nutritional, medical (cardiovascular diseases and diarrheal diseases), religious (festive periods, Christmas for Christian and Salah for Muslims,

New Year celebration) and social uses (weddings, birth celebrations, and funeral wakes).However, their effect on diabetes when consumed orally has not been scientifically claimed. Hence there is need to isolate and characterize probiotic lactic acid bacteria that could be present in palm wine and the effect of palm wine consumption by diabetic patient by assessing some diagnostic parameters.

Material and Methods

Sample Source and Inclusion and Exclusion Criteria

Palm wine of two different producers which are original and not expired was selected for the research study.

Sample Collection

The palm wine was collected into a universal sterile container observing universal sterile technique.

Sample Processing, Phenotypic Diagnosis and characterization of Lactic acid bacteria

Sample processing

Halfmillilitre(0.5ml) of the palm wine from two different producers was collected from the containers and serial dilution of $10^{-2}$of the two different brands of palm wineusing sterile water solution (Macroxpress^{R}, a division of Tumetric Diagnostics Ltd.) and incubated at 25^{o}C for 30 minutes. Streak plating method was used as described by Mohan &Murugalathawith some slight modification (Mohan &Murugalatha, 2011). Then 50µL from each dilution of the samples was inoculated and cultured into de Man, Rogosa, and Sharpe (MRS; TITAN Biotech Ltd., Rajasthan, India) agar using micropipette and incubated at 37^{o}C for 72 hours anaerobically to obtain different colonies and then purified by streak plate techniques on MRS agar for subculture and incubated at 37^{o}C for 72hours anaerobically again to obtain pure culture. The pure colony from each sample was further inoculated into MRS broth containing 12% glycerol and immediately subjected to DNA and sequencing. Finally an in-vivo evaluation was carried out using wistar rat (Nabi *et al*., 2020).

Phenotypic Diagnosis of Presumptive LAB

Pure culture of the isolate obtained from the palm wine drink was assessed for their colonial morphology and further subjected to gram stain reaction, biochemical test (oxidase, catalase and

carbohydrate fermentation test) using Mohan and Murugalatha method for identification of presumptive lactic acid bacteria (Forhad *et al*., 2015). For confirmation of the isolate as Lactic acid, it was further subjected to temperature and sodium chloride tolerance test. A loop full of suspected isolate was inoculated into 8mls of MRS broth and examined for their ability to grow at different temperatures (12, 37, 46^{o}C) and saline concentration (2.5, 4.5 and 6.5% (w/v) NaCl) for 72hours and then incubated at 37^{o}C anaerobically. Both procedures were described by Davodabadi and his colleagues in 2015 by observing the level of turbidity rate of the isolate. Immediately after the isolates where phenotypically identified and confirmed as lactic acid bacteria, they are then subjected to molecular identification immediately. Some of the pure isolate remaining are stored in MRS broth containing 15% of glycerol at -10^{o}C prior further analysis (Davodabadi*et al*., 2015).

Molecular Identification of the Isolated Presumptive Probiotic LAB

The extraction of the Deoxyribonucleic acid, quantification, amplification, purification and sequencing of the isolate were done using extraction kit on ZR fungal/bacterial DNA mini prep (Inqaba in South Africa), Nanodrop spectrophotometer, polymerase chain reaction (template of 100 base pair), agarose gel electrophoresis technique and 3510 ABI sequencer respectively (Anna *et al*., 2017).

Characterization of Functional and Safety Properties of Confirmed Probiotic LAB Isolates

Acid Tolerance Assay

The isolate obtained from 24hours culture in MRS agar was cultured for 72hours in 5ml of MRS broth at 36^{o}C and settled by centrifugation for 10min at 6000rpm. The precipitate was modified withone normality of hydrochloric acid to obtain a pHof 2.5 and then incubated anaerobically at 36^{o}C for 2 hours and assessed for growth by assessing the turbidity. To further evaluate the resistant of confirmed isolate to acid, the methodology used by Rajesh and his colleagues was applied with slight modification where fifty microliters (50µL) of the bacteriawas inoculated in MRS agar in duplicates and examined after 72 hours of incubation anaerobically at 37^{o}C. The inoculated plate which showed growth of more 10^{6} CFU/ml (number of colonies of bacteria in each plate was between 5 and 20) were considered as resistant to acid (Rajesh *et al*., 2016).

Bile Tolerance Assay

Bile tolerance was evaluated using the method used by Rajesh and his colleagues in 2016 with much modifications where fifty microliters 50µL of bacterial culture of the isolate of confirmed

LAB isolate was transferred to 5mL of modified MRS broth containing 0.15 and 0.3% bile salt concentration and then incubated at 37^{o}C anaerobically and viable growth on MRS broth was assessed for turbidity after 0, 4 and 24hours and their ability to reduce the pH (pH meter, Mimyotech instrument, Tunsia) of the modified MRS broth solution at 0, 4 and24hours of incubation The isolate showing turbidity and reduce the pH of the modified MRS broth is considered to be resistance to bile salt (Rajesh *et al*., 2016).

Safety Assay

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was carried out with Muller Hinton agar diffusion by Kirby Buer method (phenotypic methods) using commercially available antibiotic discs (MAST, Berkshire, UK) in Nigeriaas described by Estifanos in 2014. After streaking, antibiotic discs of gram positive disc was placed on the inoculated MHA agar surface using sterile forceps and then incubated at 37^{o}C for 72 hours under anaerobic condition, after which the inhibition zone diameters was measured with vernier caliper and results was interpreted (Vijayakumar *et al*., 2015). *Lactobacillius acidophilus* were used for positive quality control.

Hemolytic Test

To assess the safety via hemolytic testing, Yasmin and his colleague's method in 2020 was used. The isolate was streaked on Human blood agar and incubated at 37^{o}C for 48 hours in an anaerobic condition. Hemolytic activity of the isolate was examined for it type (alpha, beta and gamma hemolysis). Probiotic LAB undergoes gamma-hemolysis (Yasmin *et al*., 2020).

In-vivo Evaluation of Honey for itsAnti-diabetic Properties

Ethical consideration and study design

The rules guiding the use of animal for experimental study were adhered to after ethical consent was granted. Twelves(12) adult male wistar rats (*Rattus norvegicus*) were randomly divided into two (2) main groups. The two main groups are normal group and diabetes induced group. The diabetes-induced group was further subdivided into two (2) subgroups namely diabetic group on treatment and those not on treatment.

Management of Experimental Animals

The rats were maintained under standard laboratory conditions in the animal house. The two anthropometrical parameters assessed are Body mass index (BMI) and Lee index. The formula used for calculating the basal mass index and lee index are given below.

$$\text{Body Mass Index} = \frac{\text{Body weight (g)}}{Length^2(cm^2)}$$

$$\text{Lee}'\text{s Index} = \frac{\text{Cube root of body weight (g) x 1000}}{Nose - to - anus\ length\ (cm)}$$

Obesity is estimated in rat using BMI and lee's index. The normal BMI for rat is between 0.45g/cm^2 and 0.68g/cm^2 while the normal Lee's index for rat is less than 300g/cm(Novelli *et al*., 2006).

Parameters evaluation and Treatment of Animals

The anthropometric parameter (basal mass index and Lee's index), biochemical parameters (blood glucose, total cholesterol) and hematological parameter (packed cell volume) was estimated before and seven (7) days after Streptozotocin administration. The standardized dose of streptozotocin injection used to induce diabetesis 68mg/Kg body weight The male adult wistar rats with blood glucose of greater or equal to 185mg/dL and total serum cholesterol level of greater than or equal to 105mg/dL; and change in body weight via assessment of their BMI/ Lee index (5% decrease of initial weight). Blood samples were collected from the tail vein at 0, 4, 6, 8 and 10thweeks for estimation of the parameters (Rajesh *et al*., 2016). The treatment where divided into two groups namely:

Group A: Normal Study. The rats in this group was fed with normal rat feed and water orally daily via gastric gavage but where not induced with Type-2 diabetes.

Group B: Diabetes-Induced Study

Group B subgroup I: Control. The rats in this group are diabetic-induced and were given commercial feeds and treated with 0.9% physiological saline (3mL/Kg/BW) orally daily via gastric gavage. They serve as positive control (Rajesh *et al*., 2016).

Group B subgroup II: Palm wine (3mL/Kg/BW). The rats in this group are diabetic-induced and were given commercial feeds and treated with palm wine (3mL/Kg/BW) orally daily via gastric gavage to also assess the glycemic index of palm wine. Each parameter were estimated using appropriate device and instruments such as glucometer (Accu-check) and automated total cholesterol machine (Accu-answer)

Statistical Analysis

All the measurements were performed in duplicates and the results (data) were expressed as mean ± standard deviation (SD). Data were analyzed by the use of two-way analysis of variance (ANOVA, SPSS 17.0) test.

Results and discussion

Table 1 showed that out of the two (2) different honey sources used, only one (Fresh locally made palm wine drink) of them (J_2) grown on MRS agar after incubation anaerobically at 37^{o}C for 48hours of incubation.

Table 1: Isolation of presumptive Lactic acid producers from samples collected

Market (s)	Fermented food (s)	No of colonies	No. of isolates
Market square, Yenagoa.	Industrially made palm wine	0	Nil
Imirringi road, yenagoa	Fresh locally made palm wine	2	J_1, J_2
Total	2	2	2

The isolate obtained was subjected to phenotypic identification. This is illustrated in plate 1 and figure 1. The isolate was confirmed gram positive bacteria with cocci shape, catalase-negative, oxidase-negative and sugar fermenters (lactose and dextrose).One of the isolates was a fungi, while the other was confirmed bacteria after gram staining was done. The isolate is tolerant to temperature of 12^{o}C, 37^{o}C and 46^{o}C and saline of 2.5%, 4.5% and 6.5% as showed in table 2. The bacterium did not grow at a temperature of 12^{o}C, but rather grew optimally at a temperature of 37^{o}C and 46^{o}C. The isolate grew optimally at a saline concentration of 2.5%, 4.5% and 6.5% that's has been incorporated into MRS agar.The DNA sequencing and agarose gel electrophoresis done, shows that the isolate obtained is *Weissellaconfusa*.

Table 2: Microscopic, biochemical and physiological characterization of Lactic acid Bacterium isolated

Tests/Isolates	*Weissellaconfusa*
Morphology	Cocci
Biochemical testing	

Catalase	Negative
Oxidase	Negative
Carbohydrate fermentation	
Glucose	Positive
Lactose	Positive
Physiological growth at different temperature	
12^{o}C	Negative
37^{o}C	Positive
46^{o}C	Positive
Growth at different NaCl concentration	
2.5%	Positive
4.5%	Positive
6.5%	Positive

The sequence homologies detected through phylogenetic analysis showed that the isolate *Wissellaconfusa*has 100% similarity with an already previously identified EU157913*Weissellaconfusa* from the gene bank.

Table 3, 4, 5, and 6 showed the tolerance of the confirmed LAB to acid and bile salt. Table 3 shows the susceptibility of *Weissellaconfusa* cells after being inoculated in MRS broth modified with hydrochloric acid of pH 2.5 and bile salt at a concentration of 0.15 and 0.3%.

Table 3: Probiotic characteristics (acid and bile tolerance test) of *Enterococcus fecalis*

Inhibitory condition/ LAB isolates	***Weissellaconfusa***
Acid tolerance test	+
pH 2.5 at 0hour	+
pH 2.5 at 24hours	+
Bile tolerance test	+
0.15% bile at 0hour	+
0.15% bile at 6hours	+
0.15% bile at 24ouhrs	+
0.15% bile at 48hours	+
0.3% bile at 0hour	+
0.3% bile at 6hours	+

0.3% bile at 12hours	+
0.3% bile at 24hours	+
0.3% bile at 48hours	+

T**able 4** showed that *Weissellaconfusa* isolated from fresh locally made palm wine had bacterial count (no bacterial growth) after its inoculum from the modified MRS broth with a pH of 2.5 after 24hours was inoculated into MRS agar and incubated anaerobically at 37^{o}C for 48hours.

Table 4: Acid tolerance test of *Enterococcus fecalis* on MRS agar after incubation in MRS broth modified with acid

Isolates	Log_{10}CFU/mL Bacterial count at 0hour ($\times10^7$)	Log_{10}CFU/mL Bacterial count at 24hours ($\times10^7$)
Weissellaconfusa	7.74±0.57	8.06±0.03

A mean ± standard deviation of 0.00 ± 0.00 signifies that those isolates had no growth (no bacterial count).

Table 5 and 6 showed the results of the analysis of pH values of the inoculated MRS supplemented with 0.15 and 0.3% (w/v) bile salt interpreted via the use of the mean or standard deviation. In MRS without bile, the inoculum (*Weissellaconfusa* cell) grew faster than MRS supplemented with 0.15% and 0.3% bile at every sampling time (0, 4 and 24 hours) with growth appearing after 24hours of inoculation. *Weissellaconfusa* showed attribute in lowering the pH of the MRS agar that has been supplemented with 0.15% and 0.3% bile salt medium when compared with at 0 hour and 24hours of inoculation.

Table 5: Tolerance to 0.15% bile salt of *Weissellaconfusa*

Culture media/ Sampling time (hours)	**MRS agar + 0.15% Bile salt**		
	0	**4**	**24**
Weissellaconfusa	5.70±0.01	5.66±0.00	5.20±0.01

Table 6: Tolerance to 0.3% bile salt of *Weissellaconfusa*

Culture media/ Sampling time (hours)	**0**	**4**	**MRS agar + 0.3% Bile salt 24**
Weissellaconfusa	5.70±0.00	5.65±0.01	5.10±0.03

Table 7 showed the antibiotic susceptibility testing (which was carried out with Muller Hinton agar diffusion with Kirby Buer method) of *Weissellaconfusa* that was assessed through determination with minimum inhibitory concentration (MIC) of ten common (10) antibiotics. The ten antibiotics include amoxicillin (AML, 20µg), norfloxacin (NB, 10µg), streptomycin (S, 30µg), levofloxacin (LEV, 20µg), chloramphenicol (CHL, 30 µg), ciprofloxacin (CIP, 10µg), rifampicin (RIF, 20µg) erythromycin (ERY, 30µg), ampiclox (APX, 20µg) and gentamicin (CN, 10 µg). *Weissellaconfusa*cells were all susceptible to all the ten antibiotics.

Table 7: Antimicrobial susceptibility test of the confirmed *Weissellaconfusa*

Antibiotics	**Disc potency (µg)**	**Isolates *Weissellaconfusa***
Amoxicillin	20	S
Norfloxacin	10	S
Streptomycin	30	S
Levofloxacin	20	S
Chloramphenicol	30	S
Ciprofloxacin	10	S
Rifampicin	20	S
Erythromycin	30	S
Ampiclox	20	S
Gentamycin	10	S

Where inhibition zone diameter

≤ 8mm= Resistant (R)

8−10mm= Moderate susceptibility (M)

≥10mm = susceptibility (S)

Finally hemolytic assay test confirmed that this strain of *Weissellaconfusa* isolated from locally made palm wine drink was gamma hemolytic. These means they are safe for consumption by human.

Table 8 shows the effect of palm wine on some anthropometric (weight, body mass index and Lee's index), biochemical (total cholesterol, fasting blood glucose), hematological (Packed cell volume) at five (5) weeks.

The FBG of T2D rat fed with honey had significant increase in their FBG (from 347 to 165.1mg/dL) compared to normal (non-diabetic) (82.7 to 85.0mg/dL) rat and T2D induced rat (222 to 301mg/dL). The weight and Total cholesterol level of T2D rat fed with honey decreased significantly. The PCV of those T2D rat fed with palm wine increased when compared toT2D induced rat (45.5 to 45.1%).

Table 8: Comparing some Anthropometric, biochemical, and hematological parameter of normal, control and treatment group

Parameter (s)	**Experimental group**					
	Normal group		**Control group**		**treatment group**	
	0week	**5weeks**	**0week**	**5weeks**	**0week**	**5weeks**
APM						
Wt(g)	205.8±31.3	200.6±13.77	229.8±1.70	222.8±13.83	213.8±5.2	191±1.90
BMI						
(g/cm^3)	0.55±0.03	0.59±0.01	0.54±0.01	0.5±0.06	0.54±0.09	0.47±0.08
LI (g/cm)	303.3±2.5	341.6±3.51	300.6±6.0	288.6±12.4	304.4±17.6	291.6±18.1
BCM						
FBG (mg/dL)	82.7±8.2	85.0±5.1	222±96.6	301.±27.9	347.4±40.6	165.1±28.9
TC (mg/dL)	90±7.0	92.2±2.1	125.8±10.5	145.8±12.83	85.1±12.4	79.5±9.7

HEM.

PCV (%)	60.8±4.1	61.7±23.08	61±2.6	54.5±5.05	45.5±1.5	45.1±4.3

Key:

APM= Anthropometric parameter; Wt = Weight ;BMI= Body mass index ;LI= Lees index

BCM= Biochemical parameter ;FBG= Fasting blood glucose ;TC= Total cholesterol

HEM= Hematological parameter ; PCV= Packed cell volume; %= percentage

mg/dL=milligram per decilitre ; g/cm= gram per centimeter; g/cm^3

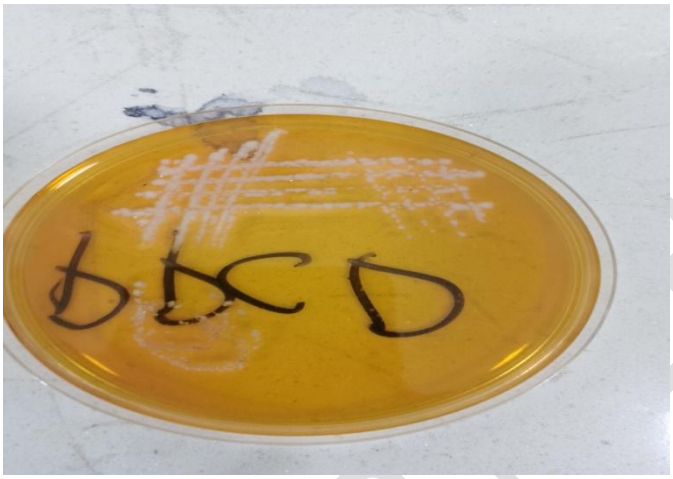

Plate 1.: Showing the growth of *Weissellaconfusa* on MRS agar

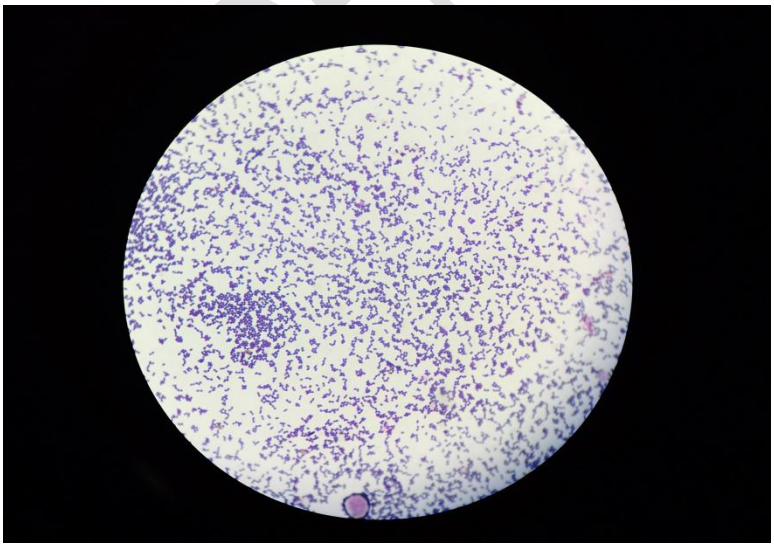

Figure 1: Showing *Weissellaconfusa* on gram stain

Discussion

Only 1 (50.0%) of the palm wine drink out of two different brands (100%) used had growth on MRS agar. The reasons why the industrially manufactured palm wine brand used in this study did not grow on MRS agar, could be due to some physical factors that can affect probiotic survival such as temperature, temperature of fermentation and pH according to Abdul *et al*. (2022). Also industrially made palm wine have been heated at a very high temperature and some additives must have been added to prevent or reduce the process of fermentation. Through this process, some of the probiotic must have been removed via their method of production in order to reduce their fermentation rate prior consumption. The bacterium isolated fit the classification of lactic acid bacteria (LAB) as gram positive, catalase-negative, oxidase-negative and carbohydrate fermentation-positive according to Akalu and his colleagues. Akalu *et al*.(2017) have also reported that the probiotic lactic acid bacteria can be isolated from fermented food product.The isolate obtained in this study grew well at 37^{o}C and 46^{o}C after incubation period of 24hours which is the optimum temperature range for their growth. However, there was no growth of these isolates at 12^{o}C. This is similar to the study of Ayodeji *et al*. (2020). The isolate was also found to grew well at 4.5 % and 6.5% salt concentration. Lactic acid bacteria should be able to survive 1.0 to 0.9% sodium chloride concentrations ideally. This feature enables them been relevant in the food industries and preservation (Shehata *et al*., 2016).

After, Polymerase chain reaction techniques was done, the Amplified DNA that wassent for DNA sequencing revealed that the isolate identified is *Weissellaconfusa*and the results were compared with the ones in Genebank database using local alignment search tool (BLAST) for complete identification with percentage prevalence of 31.58%.

*Weissellaconfusa*is among the generally regarded as safe (GRAS) microorganisms used in dextran production by various industries such as food, pharmaceuticals and clothing industries. This isolates are also similar to the bacteria strains isolated from raw milk by Yin *et al*. (2020) where heisolated and identified species of *Weissella* (*Weissellasagaenesis*) from Chinese yogurt

which was not isolated in this study. The bacteria isolated and subjected to a pH of 2.5 at 0 and 24hours showed tolerance to pH of 2.5 for 24hours.Chan *et al*.(2011) reported that acids such as the hydrochloric acid (HCl) found also in human stomach, disrupt the biomolecules of cells, such as fatty acids , proteins and DNA. Low pH environments can inhibit the metabolism and reduce the growth and viability of lactic acid bacteria.This is in accordance and consistent with the findings of Liong and Shah (2005) which stated that resistance at pH of 3 was set as standards for acid tolerance of probiotic culture.

According to Food and Agriculture Organization guidelines on safety evaluation, one of the criteria for selection of a microorganism as probiotics is that such microorganism must be safe for consumption. This is done by subjecting the microorganism to assess their hemolytic activity. The bacterium isolated from palm wine is found to be sensitive to the entire antibiotic used for antimicrobial susceptibility testing.This is not in accordance with the work of Casado and his colleagues in 2012. Casado and his colleagues in 2012 stated that some lactic acid bacteria isolated from some of fermented and dairy product possess some plasmid which might attributes to their resistance to some antibiotic. The possession of this plasmid by the lactic acid bacteria might be responsible for their intense virulence because the presence of the plasmid is responsible for the transfer of antimicrobial resistance gene via horizontal gene transfer (antibiotic resistance) to other LAB. Also lactic acid bacteria incorporated into food and drugs should not have hemolytic activity as this will indicate that they are virulent. The bacterium isolated in this study is gamma-hemolytic and as such they were not selected for other tests since their safety were confirmed. This is in accordance with the work done by Olufemi *et al*. (2018) where most LAB strains they isolated were gamma-hemolytic.

The outcome of the effect of palm wine on the T2D rat is in agreement with the outcome of Ochuko and his colleagues (2019) were they discovered that palm wine intake by diabetic rat modulates glucose homeostasis by enhancing insulin secretion as well as inhibiting redox imbalance in diabetic rats.

Conclusion and recommendation

In conclusion, palm wine is found to contain *Weissellaconfusa*and it is also found to lower or decrease the glycemic index of wistar rat that has been induced with diabetes. Based on this

present study, it is recommended that diabetic patient can consume as much palm wine as they want. Palm wine should be preserved in the refrigerator to ensure survival of the probiotic they harbor which might aid in the modulation of blood glucose level in diabetic patient. It was also found to reduce body weight and total cholesterol level in the blood.

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