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Isolation, Characterisation and Evaluation of Antiulcerogenic Potentials of Probiotic Lactic Acid Bacteria Isolated from Fermented Milk and Palm Wine against Ethanol-Induced Gastric Ulcer in Mice

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Abstract

Background: Gastric ulcer is one of the most common gastrointestinal diseases with a worldwide prevalence of about 40% in the developed countries and 80% in Africa. Acid inhibitors, anticholinergics, histamine H2-antagonists and antibiotics are commonly used to treat gastric ulcer. However, the accumulating evidence for resistance to antibiotics and the side effects of antibiotics and acid inhibitors, anticholinergics and histamine H2-antagonists. Therefore, there is an urgent need for approaches to treat and prevent gastric ulcer. One alternative strategy is the use of probiotic lactic acid bacteria. **Aim of the study:** This study aimed to isolate and characterised probiotic lactic acid bacteria from palm wine and fermented milk, and to evaluate their antiulcerogenic potentials on ethanol-induced gastric ulcer in mice. **Methods:** Probiotic lactic acid bacteria were isolated from the fermented milk and palm wine using pour plate technique on MRS agar and identified using the 16S r RNA gene sequencing. For functional properties and selection, acid and bile salt tolerance were evaluated based on viable colony count on MRS agar. Two probiotic lactic acid bacteria were selected for *in vivo* studies. Fifty-four healthy young adult Balb/c mice were randomly divided into 9 groups of 6 mice each. Gastric ulcer was induced in mice using one oral dose of absolute ethanol (10 mL/kg body weight). The probiotic lactic acid bacteria (F1 and

F2) at different doses (MF3 = 9×10^8 CFU/mL, MF6 = 1.8×10^9 CFU/mL and MF9 = 2.7×10^9 CFU/mL) and omeprazole (20 mg/kg) (a reference drug) were orally administrated daily for 14 days before ulcer induction. These mice were sacrificed 1hour after induction and the stomach contents were collected for volume and pH determination. The stomachs were subjected to macroscopic, biochemical and histopathological analysis. **Results:** Among the isolates obtained, two were considered to have the best acid and bile tolerance capacity (viable count > 7.5 logCFU/ml) and were identified as *Limosilactobacillus fermentum strain BB101* (F1) and *Lactobacillus casei strain 02* (F2). Oral administration of probiotics Lactic acid bacteria F1 and F2 significantly attenuated gastric ulcer as revealed by significant reduction ($P < 0.01$) in a dose dependent manner in the volume of gastric juice and the gastric ulcer index while significantly ($P < 0.01$) increased preventive percentage and gastric pH value when compared to the negative control group. Pretreatment with probiotic lactic acid bacteria F1 and F2 significantly increased the gastric levels of reduced glutathione (GSH), superoxide dismultase (SOD) and catalase activity (CAT) respectively with a significant decrease ($P < 0.01$) in nitric oxide (NO) and malondialdehyde (MDA) level compared to the negative control group. Pretreatment with probiotic lactic acid bacteria F1 and F2 significantly prevented mice from ethanol-induced haemorrhagic damage, desquamation of epithelial lining and edema. **Conclusion:** The results of this study revealed that palm wine and fermented milk are sources of potential probiotic lactic acid bacteria, that is *lactobacillus fermentum strain BB101* and *Lactobacillus casei 02* with excellent bile and acid tolerance capacity. Also, these probiotic lactic acid bacteria exhibit gastroprotective effect on ethanol-induced gastric ulcer via antioxidant, enhance gastric ulcer healing, antacids, and anti-secretary effects.

Keywords

Probiotics, Lactic Acid Bacteria, Antiulcerogenic Potentials, Fermented Milk, Palm Wine

1. Introduction

Background

Gastric ulcer is one of the most common disorders of gastrointestinal tract and results in various complications such as bleeding, perforation, and gastric outlet obstruction [1]. Gastric ulcer is believed to occur due to an imbalance between aggressive (HCl and pepsin) and protective factors (prostaglandins, mucus and bicarbonate barrier) in the stomach and is typically characterised by neutrophil infiltration, different stages of necrosis, blood flow reduction, increased oxidative stress and inflammation [2]. Etiological factors of gastric ulcer include alcohol abuse, smoking, stress, particularly the non-steroidal anti-inflammatory, drug overuse, and infection by *helicobacter pylori* [1] [3]. Among these factors,

high alcohol consumption is the greatest cause of gastric mucosal damage [4]. Gastric ulcer is one of the most common gastrointestinal diseases with a worldwide prevalence of about 40% in the developed countries and 80% in Africa [3]. In Cameroon, the prevalence of *helicobacter pylori* is about 64.39%, which is one of the leading causes of gastric ulcer and with a multidrug resistance rate of 70% [5] [6]. The increased drug resistance to *helicobacter pylori* has posed a serious problem in the management of gastric ulcer.

Despite the discovery of new drug, inhibition of gastric acid secretion, proton pump inhibitors, anticholinergics, histamine H2-antagonists and eradication of *Helicobacter pylori* by antibiotics have been the focus of gastric ulcer therapy [1] [7]. Furthermore, clinical evaluation of these drugs has shown incidence of relapses, some adverse effects, drug interactions, microbial resistance and high cost during chemical therapy [8]. In addition, acidity is only one component of ulcer disease and elevation of antioxidant defenses, reduction of leukocyte induced lesion may also be an important target in the preservation of mucosal integrity [9] [10]. Hence, this has drawn attention to the possible use of probiotics in the prevention and treatment of gastric ulcer with better effectiveness and safety as substitute for chemical medications. Probiotics are live microorganisms that when administered in adequate amounts confer health benefits on the host [11] [12]. Currently the available experimental and clinical studies indicate that probiotics are promising for future applications in the management of gastric ulcers. Probiotics play an essential role in the treatment or prevention of gastric ulcer induced by acetic acid, ethanol or stress, *H pylori*, NSAIDs, such as aspirin or indomethacin [13].

Fermented milk is a traditionally fermented food widely produced and consumed in the northern and western highlands regions of Cameroon. This fermented milk contains bacteria have proven to be safe and produce a wide range of antimicrobial substances such as bacteriocins and immunomodulatory properties [12]. Palm wine is a fermented traditional beverage consumed in many parts of the world and in Cameroon. Palm wine is a rich source of LAB and has proven to have a wide range of antimicrobial substances such as bacteriocins and immunomodulatory properties [12]. Both fermented milk and palm wine are commonly used to treat and prevent inflammatory related disease like gastric ulcer. In this context, they are few definite information about the gastroprotective mechanism of probiotics against ethanol-induced gastric ulcer. To explore the gastroprotective mechanism of probiotics against ethanol-induced gastric ulcer. Therefore, the is a need to isolate, characterised and to evaluate the gastroprotective potentials of probiotic lactic acid bacteria isolated from palm wine and fermented milk against ethanol-induced gastric ulcer.

2. Materials and Methods

2.1. Probiotic Bacterium

Probiotic bacterium was isolated from traditionally fermented milk and palm

wine were randomly purchased from the Bororo (Fulani ethnic group in Cameroon) around the locality of Garoua and in molyko, Buea respectively, identified by phenotypic method (gram test, catalase and biochemical) and sequencing of the 16SrRNA gene.

2.2. Experimental Animals

Balb/c mice (20 - 26 g), 6 - 8 weeks old of both sexes purchased from Laboratoire National Vétérinaire, (LANAVET), Cameroon, were used for this study. They were housed in animal house with free access to water (ad libitum) and food (food standard), 12/12 h light/dark cycle and temperature (25˚C). Mice were acclimated into the experimental environment for five days before the experiments were started. All animal experiments were carried out in accordance with national (No. FWA-IRB00001954) and international (NIH Publication 8023, revised 1996) principles of laboratory animal care.

2.3. Isolation and Identification of Probiotic Lactic Acid Bacteria from Traditional Fermented Milk and Palm Wine

The bacteria were isolated following aseptic laboratory procedures. The pour plate technique was used to isolate the microorganisms. One millilitre of each milk and palm wine sample was serially diluted up to the ten logarithmic fold in sterile test tube containing 9 mL of 0.85% saline solution (Nacl). Demann Rogosa and sharpe (MRS, Sigma-Aldrich, Germany) agar, a selective medium for lactic acid bacteria isolation was measured and prepared according to the manufacturer's instructions, sterilized and allowed to melt at room temperature. One mL aliquot of three different dilution factors (10^{-4}, 10^{-6} and 10^{-8}) were poured into plates and about 20 mL of the selective medium was added and allow solidifying at room temperature. The plates were sealed with parafilm and incubated at 37˚C for 24 - 48 hours under anaerobic conditions. After incubation, streaking technique was used to purify the colonies. The catalase negative isolates were selected and sub cultured on fresh MRS agar. A code was given to each isolate and they were microscopically examined for their catalase and Gram reactions. Gram positive bacilli/cocci and catalase negative isolates were considered as presumptive lactic acid bacteria and were kept at 4˚C in 1.5 mL Eppendorf tubes containing MRS broth for further investigation.

2.4. Functional Characterisation and Safety Evaluation

The isolates were tested for acid and bile tolerance using the methods described by Kavitha and Devasena (2013) [14]. Resistance to low pH; for entering into the upper intestine probiotic bacteria must survive against stomach pH [15]. For this purpose, active cultures (incubated for 16 - 18 h in MRS broth) were used. Cells were harvested by centrifugation for 10 min at 5000 rpm and 4˚C. Pellets were washed once in phosphate-saline buffer (PBS at pH 7.2). The cell pellets were resuspended in 5 mL of MRS broth whose pH had been adjusted (pH 2) using 1N HCL, then incubated at 37˚C for 3 hours. 1 mL of each of the suspensions

were serially diluted up to the ten logarithmic fold and the viable microorganisms (10^{-6}, 10^{-7} and 10^{-8} diluted factor) were enumerated on MRS agar after incubation at 37˚C for 0 and 3h reflecting the minimum and maximum time which food spends in the stomach. Isolates that exhibited final counts ≥ 10^3 cfu/mL or ≥10^6 cfu/mL at high pH for 4 hours, were considered to have moderate or good resistance respectively [15].

Resistance to bile salt was evaluated based on viable colony counts on MRS agar in triplicates after incubation at 37˚C for 0 and 4 h, reflecting the minimum and maximum time which food spends in the intestine. The intestinal bile concentration is believed to vary from 0.2% to 0.5%. LAB isolates were cultured in MRS broth, for 24 h at 37˚C. The cells were harvested by centrifugation for 10 min at 5000 rpm and 4˚C. Pellets were washed once in phosphate-saline buffer (PBS at pH 7.2), then suspend into 1mL MRS broth containing 0.3% (w/v) oxgall-bile (Sigma-Aldrich) respectively. Broths without oxgall-bile serve as control. 1 mL of each of the suspensions were serially diluted up to the ten logarithmic fold and the viable microorganisms (10^{-6} and 10^{-8} diluted factor) were enumerated on MRS agar after incubation at 37˚C for 0 and 4 h. Isolates that exhibited final counts ≥ 10^3 cfu/mL or ≥10^6 cfu/mL at low pH for 3 hours, were considered to have moderate or good resistance, respectively.

Safety of the isolated lactic acid bacterium was evaluated by testing their haemolytic activity. A bacterium having probiotic properties should not have haemolytic activity. This test was performed in isolated Lactic acid bacterium (LAB) with tryptic soy agar (TSA) (Sigma-Aldrich, Germany), containing 5% (w/v) sheep blood according to the method described by Schmitt *et al.* (2012) [16]. Isolates that formed a green zone around the colony were designated as alpha haemolytic while those that formed a clear zone were denoted as beta haemolytic and those that formed no zone were denoted as gamma haemolytic. *Staphylococcus aureus* strains were used as positive control. The assay was repeated in triplicates.

Safety of the isolated lactic acid bacterium was also evaluated by testing their antibiotic susceptibility. A bacterium having probiotic properties should be sensitive to all antibiotics. To evaluate the antibiotic susceptibility of lactic acid bacteria, the modified kirby-bauer susceptibility testing technique as described by Clinical and Laboratory Standards Institute guidelines was used [17]. The fresh culture of the lactic acid bacteria was streaked densely on Mueller-Hinton agar by a sterile cotton swab. Paper discs impregnated with vancomycin (10 µg), cefotaxime (10 µg erythromycin (30 µg), ampiclox (10 µg), Ceftriazone (30 µg), doxycycline (30 µg), gentamicin (10 µg), amoxil (30 µg), imipenem (10 µg) and ciprofloxacin (5 µg) were loaded on the plate. The plates were incubated for 18 - 24 h at 35˚C ± 2˚C and the slowly growing isolates were again read after 48 h of incubation. Zone of inhibition was measured in millimetres [17]. The assay was repeated in triplicates. The isolated LAB was selected and considered as candidate probiotic based on its functional properties: resistance to bile salt, resistance to acid, absence of haemolytic activity and resistant to any antibiotics.

2.5. Molecular Identification and Phylogenetic Analysis of the Probiotic Bacterium

The total genomic DNA was isolated following the protocol of Sambrook *et al.*, (1989) with some modifications [18]. PCR amplification of the 16S rDNA gene from each sample was performed to confirm the identity of the probiotic lactic acid bacteria and the small sub unit 16S rDNA genes was amplified from the genomic DNA using universal primers: -F (5'-AGAGTTTGATCCTGGCTCAG-3) -R (5'-ACGGCTACCTTGTTAACGACTT-3). The PCR conditions were as follows: initial denaturation for 5 min at 94˚C, then 30 cycles of denaturation for 1 min 30 sec at 94˚C, annealing for 1 min 30 sec at 42˚C, extension for 1 min 30 sec at 72˚C and final extension for 7 min at 72˚C. The PCR products were separated by agarose gel electrophoresis. After electrophoresis, the gel was observed for bands; indicative of successful PCR amplification in a gel imager documentation system (BIO-RAD). The size of the band was estimated by comparing with the match on the DNA ladder to see if it falls in the range of weight of 16Sr DNA of lactic acid bacteria (1000 - 2000). The 16S rRNA sequence analysis of the PCR products was determined by Inquaba biotech (South Africa). Sequence similarity search was performed using the Basic Local Alignment search tool (BLAST) against the 16S ribosomal RNA sequence database (for bacteria and Archaea) of the National Center for Biotechnology information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. Preparation of Probiotic Lactic Acid Bacteria F1 and F2 Inoculum

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 resuspended in PBS. The final concentration of the mixture was adjusted to McFarland standard (MF) 3 (MF3 = 9×10^8), 6 (MF6 = 1.8×10^9 cfu/mL) and 9 (MF9 = 2.7×10^9 cfu/mL). Also, for probiotic lactic acid bacteria F1 treatment groups were labelled as F1MF3, F1MF6 and F1MF9 while for probiotic lactic acid bacteria F2 treatment groups were labelled as F2MF3, F2MF6 and F2MF9.

2.7. Anti-Ulcerogenic Potentials of Probiotic Lactic Acid Bacteria

2.7.1. Ethanol-Induced Gastric Lesions Model

After a 1-week acclimatisation to the environment. Fifty four (54) mice were randomly assigned into 9 groups, each comprising 6 mice: Group 1 (sham control mice); Group 2 (negative control group: ulcerated mice receive PBS orally); Group 3 (ulcerated mice pre-treated with MF3 of probiotic F1); Group 4 (ulcerated mice pre-treated with MF6 of probiotic F1); Group 5 (ethanol ulcerated mice pre-treated with MF9 of probiotic F1); Group 6 (ulcerated mice pre-treated with MF3 of probiotic F2); Group 7 (ethanol ulcerated mice pre-treated with MF6 of probiotic F2). Group 8 (ulcerated mice pre-treated with MF9 of probiot-

ic F2); Group 9 (positive control: ulcerated mice pre-treated with reference drug, 20 mg/kg omeprazole). Omeprazole and probiotic lactic acid bacteria F1 and F2 were administered orally once daily for 14 consecutive days before ulcer induction. Before the experiment commenced, mice were fasted for 24 hours but with free access to water. One hour after the last treatment (omeprazole and probiotic lactic acid bacteria F1 and F2) on the 14th day, gastric ulcers were induced with absolute ethanol (10 mL/kg body weight) in these groups of mice above except group 1 (Sham control group received vehicle (PBS) only).

These mice were sacrificed 1 hour after induction and their stomachs were immediately excised. Each stomach was opened along the larger curvature, where their contents were collected for volume and pH determination. For the examination of gastric ulcer index The gastric mucosa was washed with distilled water and examined for ulcers by magnifying lens and scoring of ulcers was made as follows; normal coloured stomach (0), red coloration (0.5), spot ulcer (1), haemorrhagic streak (1.5), deep ulcers (2) and perforation (3) [19]. The stomach tissue was excised and homogenized (10% w/v) using 0.1 M Tris buffer (pH-7.4). The contents were centrifuged (2000 × g) at 4˚C for 10 min. The clear supernatant so obtained was used for to biochemical analysis for estimation of catalase, superoxide dismutase, reduced glutathione (GSH), catalase, malondialdehyde (MDA) and nitric oxide. Gastric tissues were collected at the end of the experiments and were stored in a fixative solution to determine the preventive index, using histopathology techniques [9] [20].

2.7.2. Estimation of Gastric Ulcer Index and Preventive Index

Mean ulcer score for each animal was expressed as ulcer index. The percentage of ulcer protection was determined as follows:

$$\%\ \text{protection} = \frac{\text{control mean ulcer index} - \text{test mean ulcer index}}{\text{control mean ulcer index}} \times 100$$

2.7.3. Determination of Gastric Volumes and pH

The stomachs of mice were excised out under ether anaesthesia exactly after 1 h of ethanol induce ulcer and the gastric contents were collected. The stomachs were washed with sterile water. Both the washing and gastric contents were collected and centrifuged together at 4000 RPM for 10 min. The volumes of all the supernatants of gastric contents were measured. The pH of all supernatants was measured using a pH meter.

2.7.4. Estimation of Oxidative Stress Markers in Tissue Homogenate

The gastric tissue was excised and homogenized (10% w/v) using 0.1 M Tris buffer (pH-7.4). The contents were centrifuged (2000 × g) at 4˚C for 10 min. The clear supernatant obtained was used for to biochemical analysis for estimation of catalase, superoxide dismultase, reduced glutathione (GSH), nitrite oxide and malondialdehyde (MDA).

1) Estimation of GSH

The method used by Beutler *et al.* [21] was used to determine the GSH level in

gastric tissue. The GSH level was expressed as micrograms of reduced glutathione per mg of protein.

2) MDA Assay

Malondialdehyde (MDA), resulting from lipid peroxidation, was measured using the method by okhawa *et al.* [22] and the lipid peroxides in gastric tissue were quantified in terms of TBARS [22]. The absorbance was measured spectrophotometrically at 532 nm. Results were expressed as micromoles per mg of protein.

3) Measurement of NO

Nitric oxide (NO) concentration in gastric tissues was assayed by measuring the nitric acid reductase. based on Griess diazotization reaction [23].

4) Catalase assay

Catalase (CAT) activity was measured according to the method by Aebi [24] and the initial rate of H_2O_2 disappearance at 240 nm was used to detect catalase (CAT).

5) Superoxide dismutase assay

The method by Minami and Yoshikawa was used to estimated Superoxide dismutase (SOD) activity [25].

2.7.5. Histological Study

Gastric tissues were collected at the end of the experiments were stored in the fixative solution (10% formalin). Staining was done by using hematoxylin and eosin and analyzed under a light microscope for histopathological changes (450×) [20].

2.8. Statistical Analysis

All results were expressed as means ± SE. All the experiments were performed in triplicates. Data analysis was carryout using the software program GraphPad InStat. The data obtained were analysed by one-way ANOVA and Dunnett's test. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation and Identification of Probiotic Lactic acid Bacteria (LAB)

A total of fifteen (15) LAB cultures were isolated from fermented milk and palm wine on MRS agar. Preliminary identification of colonies was carried out on the basis of cell morphology, microscopic examination and biochemical tests. Cell morphology revealed smooth, oval, and cream white colonies on MRS agar plate. However, only six colonies were catalase negative and Gram-positive bacilli under microscopic examination and were considered as presumptive LAB.

Probiotic lactic acid bacteria, mostly delivered in a food system must be acid and bile tolerant to survive in the human gastrointestinal tract. Isolates F3, F4, F5 and F6 had viable counts between 5 - 5.95 logcfu/mL, which was significantly different ($p < 0.05$) from the control (pH2.0 at 3 h) (**Figure 1**). On the contrast,

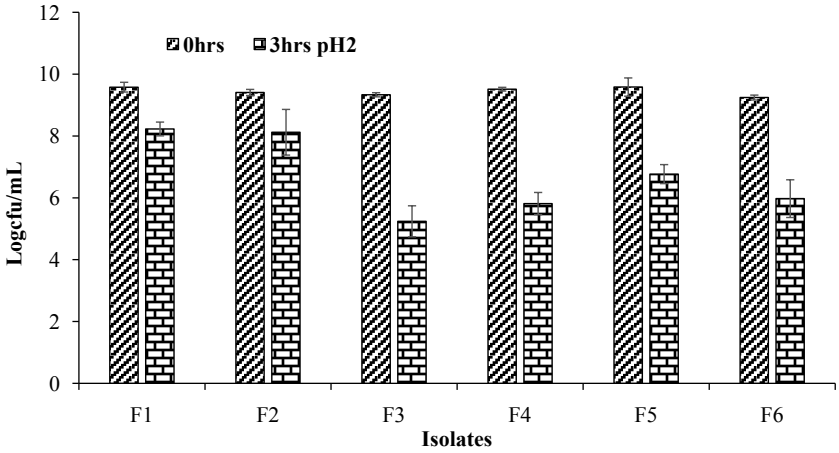

Figure 1. Acid tolerance of strains isolated from fermented milk and palm wine at pH 2 for 3 hours.

isolates F1 and F2 had viability > 7.5 logcfu/mL which was not significantly different ($p > 0.05$) when compared to the control. This implies isolates F1 and F2 has good tolerance capacity while F3, F4, F5 and F6 were moderate tolerance to low pH. Tolerance to bile salt is a precondition for colonisation and metabolic activity of bacteria in the small intestine of the host. Thus, in present study we checked the growth of lactic acid bacterial strains to bile salts of concentrations (0.3%). The viable count of isolates F1, F2, F3, F4, F5 and F6 after 4 hours of exposure to 0.3% bile salt concentration was between 5 log - 8 logcfu/mL. Also, isolates F1 and F2 exhibit the best salt tolerance viability of 7.73 logcfu/mL and 7.68 logcfu/mL respectively (**Figure 2**). The isolates F1 and F2 were negative to haemolysis test and sensitive to all the tested antibiotics thus appearing as a good probiotic. Isolates F1 and F2 were further identified by sequencing of 16 S rRNA gene. Results obtained using BLAST identification and MEGA 11 software revealed close similarity of 99.4% of F1 to *lactobacillus fermentum strain BB101* (Accession no. MF424653.1) and 99.87% of F2 to *Lactobacillus casei strain 02* (Accession no. JN560892.1).

3.2. Macroscopic Findings

The administration of absolute ethanol induces gastric ulcer along with extensive damage to the gastric mucosa with visible haemorrhagic necrosis when compared to the sham control group (**Figure 3(B)**). However, mice pre-treated with probiotic lactic acid bacteria F1 at doses F1MF3, F1MF6 and F1MF9 respectively: moderate to mild injuries are seen in the gastric mucosa, and the injuries decrease when the dose increase; hence, at F1MF9 mild gastric injuries are seen compared to the negative control (**Figures 3(C)-(E)**). Also, mice pre-treated with probiotic lactic acid bacteria F2 at doses F2MF3, F2MF6 and F2MF9 respectively: gastric mucosal damage decrease with the increase of dose; hence, at F2MF9 mild gastric injuries are seen (**Figures 3(F)-(H)**). Mice pre-treated with omeprazole (20 mg/kg), show no gastric mucosa damage and the drug completely inhibits the gastric lesions (**Figure 3(I)**).

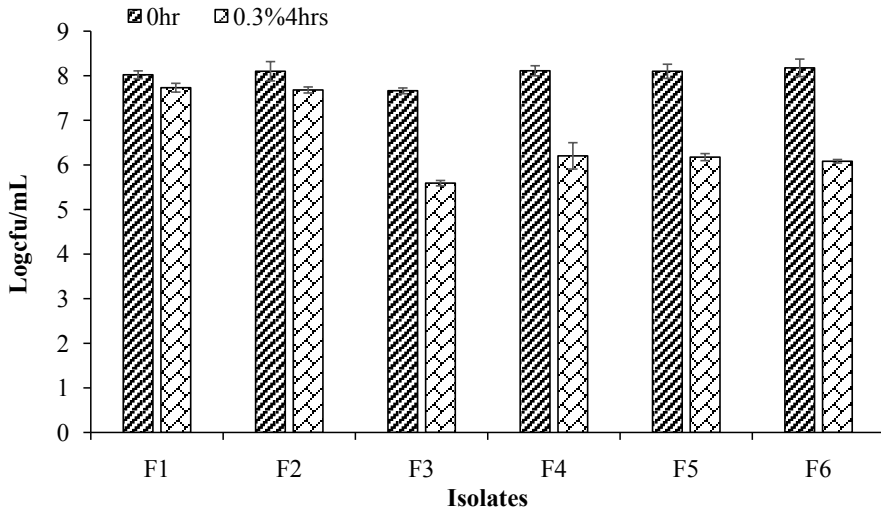

Figure 2. Bile tolerance of strains isolated from Fermented milk and palm wine at 0.3% oxgall for 4 hours.

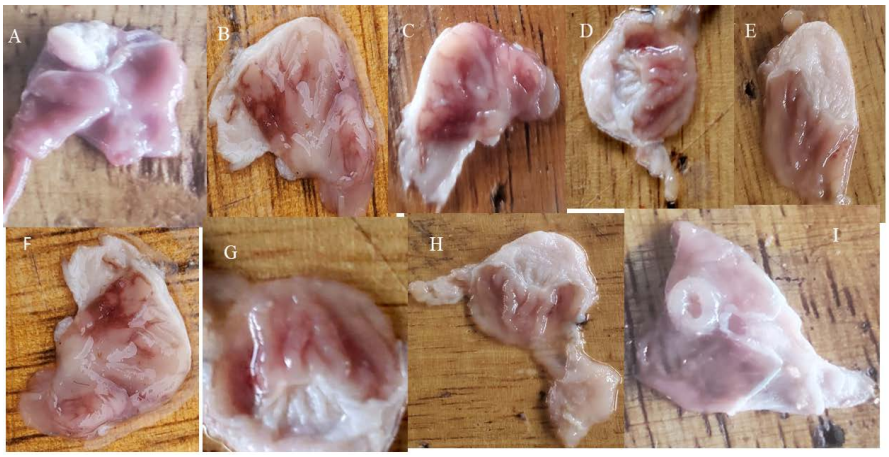

Figure 3. Photograph of mice stomach showing gastroprotective effect of probiotic lactic acid bacteria Fl and F2 on the ethanol induced gastric ulcer in mice exposed to treatment as: (A) Sham control; (B) NCG (negative control group); (C) F1MF3; (D) F1MF6; (E) F1MF9; (F) F2MF3; (G) F2MF6; (H) F2MF9; (I) OMEPR.

3.3. Effect of Probiotic Lactic Acid Bacteria F1 and F2 on Ulcer Index, Prevention Index, pH and Volume of Gastric Secretion

Oral administration of absolute ethanol induces gastric ulcer and an increase in ulcer index. Oral administration of probiotic lactic acid bacteria F1 (F1MF3, F1MF6 and F1MF9) and F2 (F2MF3, F2MF6 and F2MF9) or omeprazole prevented the development of acute gastric ulcer. Pre-treatment of mice with different doses of the probiotic lactic acid bacteria F1 and F2 exhibited significantly ($p < 0.001$) dose dependent reduction of ulcer index compared to the negative control group (Table 1). The percentage of ulcer protection of F1MF3, F1MF6 and F1MF9 of probiotic lactic acid bacteria F1 were 52.17%, 63.78% and 79.71% respectively. Also, pretreatment of ulcer mice with probiotic lactic acid bacteria F2 of dose F2MF3, F2MF6 and F2MF9 produce a percentage of ulcer protection

Table 1. Effect of probiotic lactic acid bacteria F1 and F2 on ulcer index, preventive index, pH and volume of gastric juice in various experimental groups.

Treatments	Gastric pH	Ulcer Index	% of protection Gastric Volume (mL)	
SHAM	6.91 ± 0.18	2.67 ± 0.67		0.14 ± 0.01
NCG	4.35 ± 0.22$^{###}$	23.00 ± 0.85$^{###}$		0.61 ± 0.03$^{###}$
F1MF3	5.22 ± 0.37**	11.00 ± 0.58**	52.17	0.48 ± 0.03**
F1MF6	5.52 ± 0.40**	8.33 ± 0.89**	63.78	0.37 ± 0.06**
F1MF9	6.31 ± 0.09***	4.67 ± 0.89***	79.71	0.34 ± 0.02*
F2MF3	4.65 ± 0.40*	11.27 ± 0.82***	50.00	0.54 ± 0.05**
F2MF6	4.79 ± 0.39***	8.67 ± 0.82***	62.32	0.48 ± 0.02**
F2MF9	5.44 ± 0.57***	5.00 ± 0.58*	78.26	0.50 ± 0.01*
OMEPR	6.43 ± 0.38***	4.33 ± 1.20***	81.16	0.33 ± 0.03***

Each value represents the mean ± SEM for six mice in each group. Statistically significant difference is expressed *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ Vs Negative control; $^{#}p < 0.05$, and $^{##}p < 0.01$ Vs Sham control. NCG: Negative control.

of 51%, 62.32% and 78.26% respectively in comparison to the negative control. Whereas standard drug omeprazole showed 81.16% ulcer protection (Table 1).

Ethanol administration caused significant decrease in pH value and with a corresponding significant increase in gastric volume of gastric content compared to sham control group. Treatments with probiotic lactic acid bacteria F1 (F1MF3, F1MF6 and F1MF9) produced significantly ($p < 0.001$) dose dependent increase in pH value by 20.00%, 26.90% and 45.06% respectively, and oral administration of probiotic lactic acid bacteria F2 (F2MF3, F2MF6 and F2MF9) produced significantly ($p < 0.001$) dose dependent rise in pH value by 6.90%, 10.11% and 25.06% respectively compared to the negative control (Table 1). Whereas standard drug, omeprazole showed an increase in pH by 47.81% compared to the negative control (Table 1). Pre-treatments with probiotic lactic acid bacteria F1 (F1MF3, F1MF6 and F1MF9) is associated with significant dose dependent decrease in gastric volume by 21.31%, 39.34% and 44.26% respectively as compared to negative control, oral administration of probiotic lactic acid bacteria F2 (F2MF3, F2MF6 and F2MF9) produce a significant reduction in gastric volume by 11.48%, 21.31% and 18.03% respectively as compared to negative control (Table 1).

3.4. Effect of Probiotic Lactic Acid Bacteria F1 and F2 on Oxidative Stress Markers

The effect of probiotic lactic acid bacteria F1 and F2 on oxidative stress markers that is catalase activity (CAT), superoxide dismultase (SOD), reduced glutathione (GSH), nitrite oxide (NO) and malondialdehyde (MDA).

3.4.1. Effect of Probiotic Lactic Acid Bacteria F1 and F2 on Reduced Glutathione (GSH), Superoxide Dismultase (SOD) and Catalase Activity (CAT)

The treatment of mice with ethanol significantly decreased level of gastric tissue

glutathione by 51.91% compared to the sham control group. The oral administration of probiotic lactic acid bacteria F1 (F1MF3, F1MF6 and F1MF9) and F2 (F2MF3, F2MF6 and F2MF9) or omeprazole produced a significant ($p < 0.05$, $p < 0.01$) rise in glutathione levels in the gastric tissues in a dose dependent manner. On the other hand, oral administration of probiotic lactic acid bacteria F1 and F2 at dose of F1MF9 and F2MF9 respectively in ulcerated mice significantly ($P < 0.001$) elevate gastric Glutathione level by 93.75% and 76.75% respectively compared to the negative control group. Similar effect was observed with omeprazole (Figure 4(A)). The oral administration of ethanol in vehicle treated group induce a significant reduction in the level of superoxide dismutase gastric tissue ulcerated mice by 30.77% compared to the sham group. The administration of probiotic lactic acid bacteria F1 and F2, produced a significant ($p < 0.05$) increase in superoxide dismutase levels in the gastric tissues in dose dependent manner. Interestingly, omeprazole also significantly increased ($p < 0.05$) the gastric tissues superoxide dismutase level. Oral administration of probiotic lactic acid bacteria F1 and F2 at dose F1MF9 and F2MF9 respectively, caused a notable elevation in gastric tissue superoxide dismutase level by 44.60% and 20.37% respectively

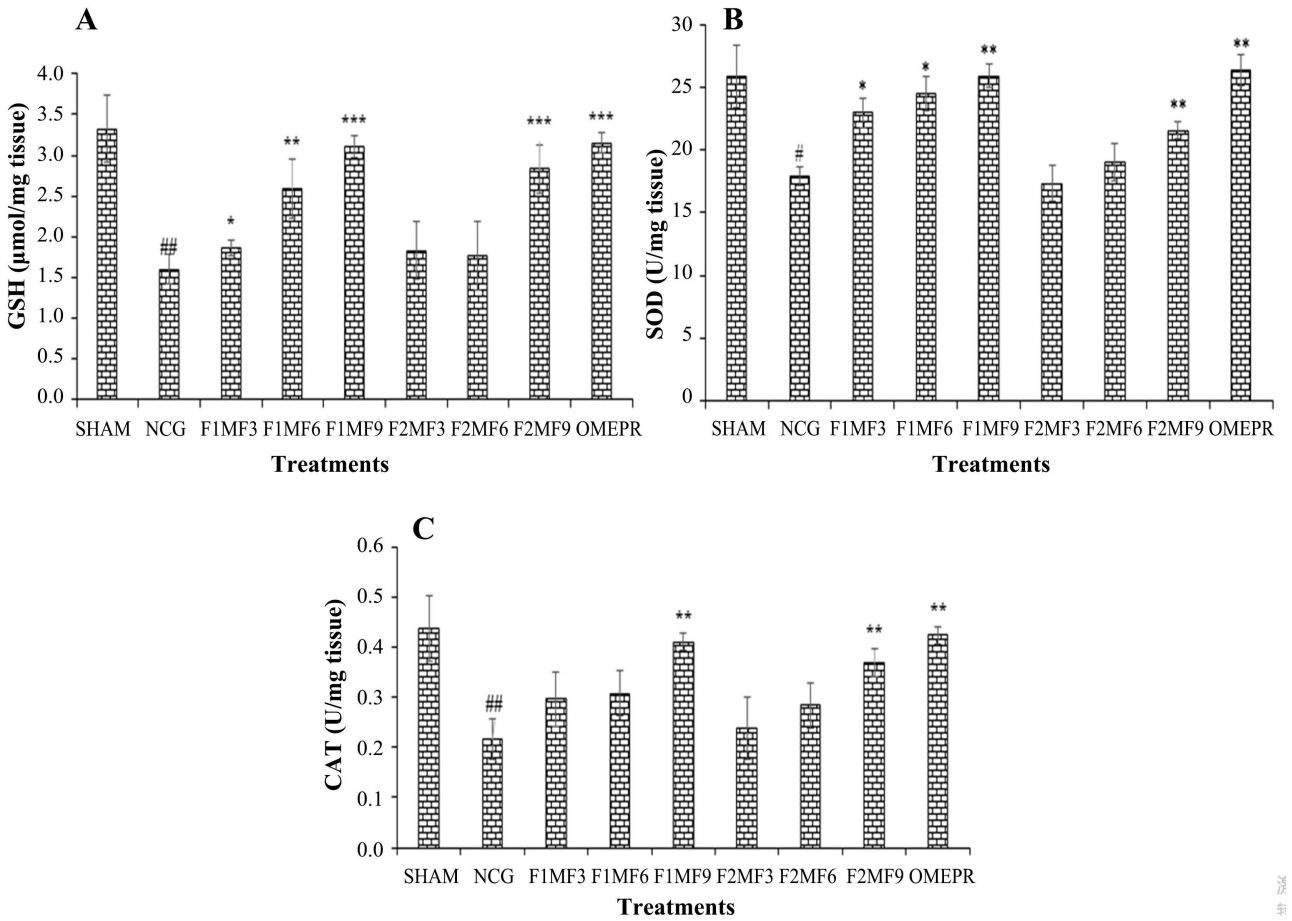

Figure 4. The Effect of probiotic lactic acid bacteria F1 and F2 on (A) GSH, (B) SOD, (C) CAT in gastric tissue of various experimental groups. Each value represents the mean ± SEM for six mice in each group. Statistically significant difference is expressed *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ Vs Negative control; #$p < 0.05$, and ##$p < 0.01$ Vs Sham control.

compared to the negative control group (Figure 4(B)). The induction of gastric ulcers with ethanol resulted in a significant reduction in catalase activity in the ulcerated mice by 49.66% compared to the sham group. When compared to negative control group, catalase activity significantly ($p < 0.05$) increased by 85.91% and 68.18% in ulcerated mice pretreated with MF9 of probiotic lactic acid bacteria F1 and F2 respectively. Also, Omeprazole also significantly increased the level of catalase activity in the Gastric tissues of mice by 90.91% compared to the negative control group (Figure 4(C)).

3.4.2. Effect of Probiotic Lactic Acid Bacteria F1 and F2 on Nitrite Oxide (NO) and Malondialdehyde (MDA)

In the present study, ulcerated mice revealed a significant rise in gastric tissue MDA as compared to the sham control. However, pre-treatment of mice with probiotic lactic acid bacteria F1 and F2 or omeprazole produced a significant ($p < 0.05$, $p < 0.01$) reduction in lipid peroxidation in a dose dependent manner in the respective groups. Also, pre-treatment of mice with probiotic lactic acid bacteria F1 and F2 at dose F1MF9 and F2MF9 respectively, significantly decreased the MDA levels in the gastric tissues by 52.6% and 49.23% respectively compared to the negative control group. The administration of Omeprazole reduced MDA levels by 55.24% compared to the negative control group (Figure 5(A)). The induction of gastric ulcers with ethanol produced a significant increase in nitric oxide level in gastric tissue in ulcerated mice by 88.10% compared to the sham group. However, pre-treatment of mice with probiotic lactic acid bacteria F1 (F1MF3, F1MF6 and F1MF9) and F2 (F2MF3, F2MF6 and F2MF9) or omeprazole, significantly ($p < 0.05$) attenuate the ethanol induce increase in nitric oxide level in gastric tissue when compared to the negative control group. Mice pre-treated with probiotic lactic acid bacteria F1 and F2 at dose of F1MF9 and F2MF9 respectively, significantly decrease the nitric oxide level in the gastric tissues by 44.05% and 29.11% respectively compared to the negative control group. Also, a significant decrease in nitric oxide level in the gastric tissues was observed in mice treated with omeprazole (Figure 5(B)).

3.5. Histological Assessment of the Effect of Probiotics Lactic Acid Bacteria F1 and F2 on Gastric Mucosal Injuries in Ethanol-Induced Mice

Oral administration of ethanol caused a significant change in the gastric epithelium including epithelial cell loss, necrotic lesions penetrating deeply into mucosa and sub mucosa layer, haemorrhagic damage and oedema with leucocytes compared to the sham control group. However, pre-treatment mice with probiotic lactic acid bacteria F1 (F1MF3, F1MF6 and F1MF9) and F2 (F2MF3, F2MF6 and F2MF9) or omeprazole before oral administration of ethanol, significantly attenuate ethanol-induced epithelial cell destruction, necrotic lesions penetrating deeply into mucosa and sub mucosa layer, haemorrhagic damage and play an essential role in the protection of the gastric wall (Figure 6). Pre-treatment of

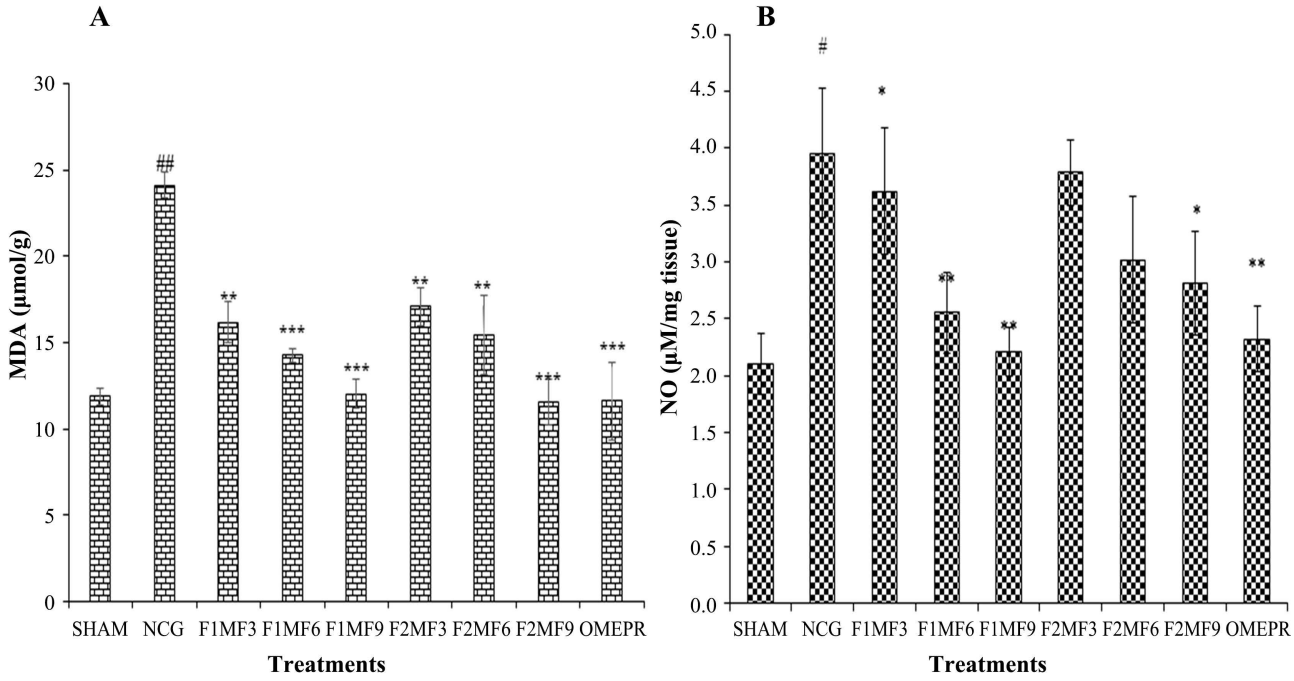

Figure 5. The Effect of probiotic lactic acid bacteria F1 and F2 on (A) MDA, (B) NO in gastric tissue of ethanol induced gastric ulcers in mice. Each value represents the mean ± SEM for six mice in each group. Statistically significant difference isexpressed *p < 0.05, **p < 0.01, and ***p < 0.001 Vs Negative control; #p < 0.05 and ##p < 0.01 Vs Sham control.

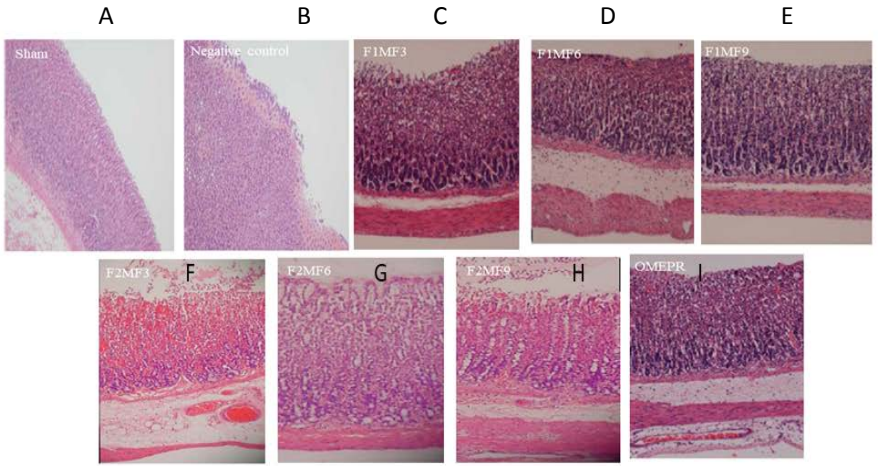

Figure 6. Histological evaluation of anti-ulcer effect of probiotic lactic acid bacteria F1 and F2 on gastric mucosal injuries in ethanol-induced mice. (A) histological section of a sham control mice; (B) Negative control; (C) F1MF3 of probiotic F1; (D) F1MF6 of probiotic F1; (E) F1MF9 of probiotic F1; (F) F2MF3 of probiotic F2; (G) F2MF6 of probiotic F2; (H) F2MF9 of probiotic F2; (I) omeprazole.

ulcerated mice with Probiotic lactic acid bacteria F1 at doses F1MF6 and F1MF9 exhibited significant regeneration and inhibited the development of haemorrhage, desquamation of epithelial lining and oedema with inflammatory cells similar effect is observe with the standard drug omeprazole (**Figure 6(D)**, **Figure 6(E)** and **Figure 6(I)** respectively). On the other hand oral administration of probiotic lactic acid bacteria F2 at dose F2MF6 and F2MF9 in ulcerated mice,

mild disruptions of surface epithelium are present but deep mucosal damage is absent and a moderate regeneration (**Figure 6(G)** and **Figure 6(H)** respectively).

4. Discussion

Gastric ulcer disease is a common disease and results in various complications such as bleeding, perforation, and gastric outlet obstruction [1]. Alcohol consumption has been regarded as the principal cause of gastric ulcer in humans. Thus, the current study was designed to study gastroprotective effect of probiotics lactic acid bacteria against ethanol-induced gastric ulcer in comparison to omeprazole, which is widely approved and used for treatment gastric ulcer.

In our study, we successfully isolated a probiotic bacterium *lactobacillus fermentum strain BB101* (F1) and *Lactobacillus casei strain 02* (F2) from traditionally fermented milk and palm wine respectively and molecularly characterized it using the 16S rRNA gene. From our study, probiotic bacterium *lactobacillus fermentum strain BB101* (F1) and *Lactobacillus casei strain 02* (F2) had a good acid and bile tolerance capacity. These results of acid and bile salt tolerance is similar to that obtained by other authors who reported that *Lactobacilli* which were isolated from milk products and palm wine showed resistance to low and high pH respectively [26] [27]. Safety is also a significant consideration while selecting potential probiotics before they are available for public usage, these strains were gamma haemolytic (no haemolysis) and sensitive to all antibiotics tested, these results is similar to previous studies [26] [27].

To evaluate the protective effects of Probiotic lactic acid bacteria F1 and F2 on gastric ulcer induced by oral administration of absolute ethanol. In the present study, a high degree of ulceration was observed in mice treated with absolute ethanol. This was clearly confirmed by macroscopic and histopathological results which show severe haemorrhage, epithelial cell destruction, necrotic lesions penetrating deeply into mucosa and sub mucosa layer and oedema with infiltration of inflammatory cells. This result is in line with mousa *et al.* [28], who reported that ethanol administration may induce gastric micro-vessel disturbance which lead to necrotic gastric injury. This result is probably due to ethanol toxicity which cause continuous haemorrhage and alterations in the level of some pro-inflammatory and inflammatory mediators. Pre-treatment of mice with probiotic lactic acid bacteria F1 and F2 significantly reduced the ulcer index at all dosage respectively compared to negative control in dose dependant manner. Also, ulcerated animals pre-treated with probiotic lactic acid bacteria F1 and F2 showed a better reduction in ulcer index and preventive index comparable to the standard drugs, omeprazole, indicating that probiotic lactic acid bacteria F1 and F2 are valuable in healing and protecting gastric ulcer. However, ulcerated mice pretreated with probiotic lactic acid bacteria F1 and F2 revealed restoration of the mucosal epithelium, few inflammatory cells and no hemorrhage from histopathological findings. This result is in line with studies, which revealed that probiotics not only inhibit the development of acute gastric mucosal lesions, but al-

so accelerate the process of healing of induced gastric ulcers [29] [30] [31]. These may be due to the ability of probiotic lactic acid bacteria F1 and F2 to upregulate the expression and production of vascular endothelial growth factor and also through the upregulation of prostaglandin E2 [30] [31].

Studies show that increase concentration of the hydrogen ion is an aggressive factor facilitating gastric damage via decreasing pH in gastric juice. The present study showed a significant decrease in gastric pH level in ethanol treated mice when compared to sham control group. Probiotic lactic acid bacteria F1 and F2 pre-treatment in ethanol-ulcerated groups significantly increase gastric pH levels in a dose dependent manner with a significant reduction in gastric secretion in comparison to negative control group. Our results are similar to a study were ethanol-induced gastric mucosal lesions in rats were prevented by pre-treatment with the probiotic strain *Lactobacillus rhamnosus GG* through the upregulation of prostaglandin E2 [31] [32]. This is probably due to the fact that Prostaglandins are involved in the ulcer healing process by inhibiting acid secretion and stimulating the production of mucus [30]. The significant reduction in gastric volume secretion from our study is probably due to suppression of histamine which concur with a study, where oral administration of Lac-B showed significant anti-allergic effect by decreasing histamine content [33].

Nitric oxide, derived from constitutive nitric oxide synthase, is an important endogenous mediator of mucosal defense and plays a significant role in the maintenance of normal gastric mucosal integrity by boosting mucus and bicarbonate secretion, regulating gastric blood flow and microcirculation, and downregulating neutrophil aggregation and secretion [30] [34]. From our study, ethanol ulcerated mice showed significant increase in gastric NO levels in comparison to sham control group, this result is similar to previous studies [28] [35]. Also, ulcerated mice pre-treated with probiotic lactic acid bacteria F1 and F2 displayed marked dose dependent decrease in NO level compared to the negative control, indicating its anti-ulcer properties. This result is in accordance with Khoder *et al.*, who reported antioxidant activities of probiotic lactic acid bacteria [29] [30]. The significance decrease in NO is probably due to the antioxidant activities of probiotic lactic acid bacteria F1 and F2. Alcohol also increases the generation of reactive oxygen species (ROS) and lipid peroxidation while suppressing the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase. These enzymes play important roles in protecting stomach against mucosa damages [36]. In our study, this was a significance inhibition of SOD, GSH and CAT activities in the ethanol ulcerated mice. This is in accordance with previous studies [34] [37]. Also, pre-treatment of ethanol ulcerated mice with probiotic lactic acid bacteria F1 and F2 increase the SOD, GSH and CAT activities in a dose dependent manner compared to the negative control. These results indicate that probiotic lactic acid bacteria F1 and F2 may play an important role in eliminating gastric damage by enhancing the activity of antioxidant enzymes (CAT, SOD and GSH-Px) thus preventing oxid-

ative damage. Increasing lipid peroxidation products, such as MDA, is often used to indicate the extent of oxidative stress. Our results show a significant increase in the concentration of MDA in the gastric tissue of ethanol ulcerated mice. However, pre-treatment of ethanol ulcerated mice with probiotic lactic acid bacteria F1 and F2 significantly reduced MDA levels in a dose dependent manner compared to the negative control group. This result is in accordance with Khoder *et al.*, who reported antioxidant activities of probiotic lactic acid bacteria [29] [30]. The significance decrease in MDA is probably due to the antioxidant activities of probiotic lactic acid bacteria F1 and F2. This result is consistent to other studies, which establish that one of mechanism of enhanced ulcer healing is by restoring the balance between pro- and anti-oxidants in the gastric mucosa [28] [38]. The antioxidant effect of probiotic lactic acid bacteria F1 and F2 may probably be due to down regulation of enzyme activities that mediate ROS production, self-secretion of antioxidant metabolites and modulation of the antioxidases activities [39].

However, ulcerated mice pre-treated with probiotic lactic acid bacteria F1 and F2 revealed restoration of the mucosal epithelium, few inflammatory cells and no haemorrhage from histopathological findings, this result is comparable to the standard drug (omeprazole). These may be due to the ability of probiotic lactic acid bacteria F1 and F2 to upregulate the expression and production of vascular endothelial growth factor and also through the upregulation of prostaglandin E2 [30] [31]. Vascular endothelial growth factor is a fundamental angiogenic factor, which stimulates formation of granulation tissue and new micro vessels via angiogenesis that in turn accelerates gastric and duodenal ulcer healing [30] [31].

5. Conclusion

The results of this study revealed that palm wine and fermented milk are sources of potential probiotic lactic acid bacteria, that is *lactobacillus fermentum strain BB101* (*F2*) and *Lactobacillus casei02* (*F2*) with excellent bile and acid tolerance capacity. Also, this *lactobacillus fermentum strain BB101* and *Lactobacillus casei strain 02* exhibit the antiulcer effects via antioxidant, antacids, enhance ulcer healing and anti-secretary effects. Therefore, *lactobacillus fermentum strain BB101* and *Lactobacillus casei strain 02* could be used as a promising anti-ulcer agent in the treatment of gastric ulcers due to its comparable anti-ulcer effect to that of omeprazole but *lactobacillus fermentum strain BB101* protect better than *Lactobacillus casei strain 02*. Therefore, the use of probiotics in the management of gastric ulcer appears promising and further studies are required.

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Authors' Contributions

NFA, TBF and SGT conceived and designed the study: TBF and NFA implement

the study: TBF and SGT supervised the study. NFA, TPB and SGT conducted data analysis: TBF, TPB, TNM, NFA, LLTT and SGT interpreted study results: NFA wrote the first draft of the manuscript, SGT, SW and TBF reviewed and corrected the manuscript. All authors approved the final copy.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Serum aspartate transaminase; CAT: Catalase; GSH: Reduced glutathione; GSH-Px: Glutathione peroxidase; H&E: Hematoxylin and eosin; MDA: Malondialdehyde; MPO: Myeloperoxidase; NO: Nitric oxide; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TNF-α: Tumor necrosis factor-alpha; LAB: Lactic Acid Bacteria.