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Immunomodulating Potential of Exopolysaccharide Producing Lactic Acid Bacteria Isolated from Fermented Milk Products

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Abstract

Vaccines obtained through immuno-chemotherapy and non-specific immune-stimulants are necessary to improve human health. Bacteria probiotics such as exopolysaccharide producing lactic acid bacteria (EPS^+ LAB) exert health benefits through immunomodulation and have received considerable attention in the dairy industry. However, information regarding EPS^+ LAB and its implication on human health is lacking. Therefore, an effective assessment of the safety and immuno-modulating potential of EPS^+ LAB is needed. The study was designed to investigate the characterisation and immuno-modulating potential of EPS^+ LAB from fermented milk products. The EPS^+ LAB cells were washed in phosphate buffered saline (PBS, pH 7.0) and 1.0 x 10^9 cfu/mL suspended in skimmed milk. One millilitre of cell suspension was orally administered to experimental Wistar rats for seven days. A control group was fed with skimmed milk without EPS^+ LAB. The safety of EPS^+ LAB in Wistar rats on haematological, serum enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total protein was assessed using standard method. Histopathology of Wistar rat organs (Kidney, Liver, Lung and Heart) was determined. The immunomodulatory activities (Immunoglobulins A and G, Interleukin-2; Gamma interferon and Alpha tumour necrotic factor) of EPS^+ LAB on Wistar rats were carried out using ELISA. All procedures followed standard methods. Data were analysed using one-way ANOVA at $\alpha_{0.05}$. EPS-producing strains *Lactobacillus plantarum* (Y2) and *Lactobacillus pentosus* G4, were identified and used in this study. Haemoglobin levels in Wistar rats administered *L. plantarum* Y2 and *L. pentosus* G4 fell within safety limit of 14 - 16 g/dL. Serum enzymes: (AST 51.67 ± 4.04 x 10^9/L - 55.67 ± 8.62 x 10^9/L); (ALT 26.67 ± 3.21 x 10^9/L - 32.33 ± 2.89 x 10^9/L); (ALP 122.50 ± 9.19 x 10^9/L - 126.00 ± 12.73 x 10^9/L); and total protein (7.30 ± 1.25 g/dL - 7.77 ± 1.63 g/dL) and albumin (3.57 ± 0.73 g/dL - 3.86 ± 0.88 g/dL) were all within normal limits. Histopathological data showed no tissue lesion, congestion and necrosis in both the treated and control rats. *L. plantarum* Y2 increased IgA levels in intestinal mucosa (1205.47 ± 50.09 pg/ml). Interleukin-2 values were significant compared to control while gamma interferon and tumour necrotic factor were not significant compared to control.

Conclusion: *L. plantarum* Y2 stimulated immunomodulatory activities at accepted safety levels in Wistar rats.

Keywords: *Exopolysaccharide-producing Lactic Acid Bacteria; Fermented Milk Products; Immunomodulatory Potential; Safety-assessment; Serum Enzymes*

Introduction

Lactic acid bacteria (LAB) comprises of a group of Gram positive, low guanine-cytosine content, acid tolerant, aero-tolerant and catalase-negative organisms that are non-respiring rod or cocci that are devoid of cytochromes and anaerobic [1]. Genera of LAB that are used in the food industry capable of secreting exopolysaccharides (EPS) into the growth medium which also produce lactic acid and other metabolites during fermentation are *Streptococcus, Lactobacillus, Enterococcus, Lactococcus, Leuconostoc, Pediococcus* and *Aerococcus* [2].

These organisms are fastidious and strictly fermentative [3] that have gained much attention because of their safety status, functional properties and biomolecules which they produced [4,5]. These organisms have and will continue to play an important role in the survival of mankind.

LAB produces antibacterial compounds that inhibit pathogenic organisms [6]. These organisms had been known to promote health by stimulating production of antibodies, phagocytic activity against pathogens in the intestine and other tissues of the body. Production of inhibitor substances other than organic acids that are antagonistic towards other microorganisms have been reported [7; 8]. LAB combat the proliferation of bacterial pathogens by competitive exclusion generally. LAB is regarded as safe without harmful effect on humans and animals thus can be a good alternative for a country that lacks adequate primary health care, indiscriminate use of antibiotics and worrisome practice of using concoctions to cure diarrhoea and other infections. Extensive and self-medication use of antibiotics inhibit or kill beneficial microbiota in the gastrointestinal (GI) ecosystem and even drug resistance among pathogenic microorganisms. This could be life threatening as non-pathogenic organisms may become pathogenic through exchange of genetic material within the intestine [9].

Thus, call for the use of EPS^+ LAB as potential immunomodulator, more so, in order to improve health and welfare of human, several alternatives through the use of vaccines, nonspecific immunostimulants, and bacterial probiotics exopolysaccharide producing LAB (EPS^+ LAB) should be encouraged. However, there is a paucity of information regarding EPS^+ LAB isolated from fermented milk and its implication on health. This research work therefore centres on the immunomodulating potential of EPS+ LAB in-vivo using Wister Rats.

Materials and Methods

Samples collection

Ten samples each of fresh milk, nono, wara (unaged traditional fermented soft cheese) and yoghurt were purposively purchased from Bodija market, Ibadan. The dairy samples were collected aseptically in sterile bottles, kept in an ice box and transported immediately to the Microbiology Laboratory of the University of Ibadan for microbiological analysis.

Isolation of lactic acid bacteria

One millimeter/one gram of fermented milk, yoghurt, nono and wara (cheese) were serially diluted [10]. Aliquots 0.1 mL of the fourth dilution was mixed with 18 mL of sterile molten MRS agar medium contained in McCartney bottles; this was followed by pour plating aseptically into sterile Petri-dishes. After setting, the Petri-dishes were incubated in anaerobic jars at 30oC for 48h. Colonies were repeatedly sub-cultured until pure cultures were obtained.

Phenotypic characterization of isolates

The isolates were initially differentiated on the basis of their cultural and cellular morphological such as growth type, shape, elevation, size, pigmentation and consistency by employing macroscopic and microscopic, after which they were subjected to various biochemical, chemical and physiological test.

Identification of the isolates

The isolates were identified based on the results of the various biochemical tests [11].

Acid tolerance test of lactic acid bacteria isolates at different pH

The method of Conway., *et al.* [12] was employed. The cultures were grown in MRS broth (Oxoid) at 37°C overnight, then subculture into 10 mL of fresh MRS broth and incubated for another 24 h. Thereafter, centrifuge at 2,000Xg for 10 minutes at 4°C and the pellets washed twice in sterile phosphate buffered saline (PBS, pH 7.0). The concentration of the suspension was determined by comparing the turbidity with Mcfarland tubes of barium sulphate standard. The number of LAB per millilitre was determined by plating 10 fold serial dilutions of the suspension on MRS agar. Titre was expressed as colony forming unit per mL (cfu mL^{-1}). For each LAB strain, 0.1 mL of culture suspension with a concentration of 3.2 x 10^{7} cfu/ mL was added separately into a series of tubes containing 2 ml of sterile PBS at various pH values of 2 - 6. The tubes were incubated for 2, 3 and 4h. The test was performed in triplicate for each strain. After the incubation period, 0.1 mL of serially diluted samples from each tube was cultured on MRS agar plates, incubated at 37°C for 48h and followed by determination of viable count.

Bile tolerance test

The method used for testing bile tolerance was described by Walker., *et al* [13]. The LAB isolates were grown overnight in MRS broth and 0.1 mL of culture suspension was inoculated into tubes containing 10 mL MRS broth with 0.3% (W/V) Oxgall (sigma) or without (which acted as controls). The inoculated tubes were incubated at 37°C. Growth was monitored two hourly for 8 h. by measuring absorbance at 540 nm using labda 25av/visible spectrophotometer (PerkinElmer precisely).

Safety assessment of the isolates

Vancomycin susceptibility test

Commercially produced vancomycin disk 30 ug (Oxoid) was used for the test. Antimicrobial susceptibility was determined using the disk diffusion method which was performed on Mueller Hinton Agar (Oxoid). The inoculum was standardized using the 0.5 McFarland turbidity standard which corresponds to 1.5 x 10^{8} cfu/mL of cells. Antibiotics discs was then placed on the plates using a sterile forceps and incubated at 37°C in a candle jar. Zone diameters were recorded after 24h incubation [14].

Haemolytic activity

Haemolysin activity was determined by streaking the test isolates on Blood Agar base containing 5% human blood, incubated for 48h at 37°C. After 48h of incubation, complete or partial clearing zones were observed indicating β and α haemolysis production [15].

Gelatinase test

Nine milliliters of 10% Gelatin broth were dispensed into test tubes and sterilized at 121°C for 10 minutes. The test organisms were inoculated into the tubes and incubated at 30°C for 7 days. After incubation, the tubes were refrigerated to solidify undenatured gelatine [16].

Investigation of the *in-vivo* immunomodulating potentials of the EPS producing LAB

Four weeks Wistar rats (160 ± 5g) were obtained from Institute for Advanced Medical Research Training, College of Medicine, University of Ibadan. Wistar rats were housed ten per cage (with sterilized wood shaving as bedding) and held for seven days before the study began for acclimatization. The animals were kept under standard conditions (temperature 2 ± 3°C, relative humidity 40% - 70%, 12h light/12h dark cycle). The experiment was performed as stipulated in the guidelines on animal research of the Animal Research Ethic of the University of Ibadan.

Preparation of inoculum

Prior to challenge, the exopolysaccharides producing LAB, *L. pentosus* G4 and *L. plantarum* Y2 were grown in MRS broth at 30°C to late logarithmic phase i.e. 48 hours. The cells were harvested by centrifugation at 3,100 x g for 30 minutes. The cells were then washed twice in 100 ml cold sterile 0.9% NaCl solution and OD_{600} was adjusted to 1.5 which corresponded to ~ 1 x 10^9 cfu/ml based on a viable plate count determination.

Toxicity test

This was used to access the effect of *L. pentosus* G4 and *L. plantarum* Y2 isolates applied in a single daily oral dose of 10^9 cfu/ml on five healthy Wistar rats for seven days.

Inoculation of Wistar rats

A total of 30 Wistar rats (4 weeks old) with weights ranged between 160 ± 5g were utilized for this experiment. They were housed in groups of 10 per cage, each fitted with a drinker and a feeding bowl. In addition to the commercially prepared feed, the treated rats (the first two groups) were orally administered with exopolysaccharides producing LAB (*L. pentosus* G4 and *L. plantarum* Y2 (109 cfu/ml)) which were suspended in 1 ml sterile non-fat (skimmed) milk with a gastric needle-tube or sterile non-fat (skimmed) milk (control group) for seven days.

Specimen collection

Blood samples (1 mL per rat) were obtained via cervical dislocation after anaesthesia. Half of the sample was collected into an ethylene di amine tetra acetic acid (EDTA) tube for blood count and the other half into a sterile tube with no anticoagulant for separation for serum biochemistry (quantitative determination of total protein, albumin globulin, glucose, packed cell volume, haemoglobin). Serum was obtained by centrifugation at 3000g for 5 minutes. The serum was collected using a Pasteur pipette. At the end of the experiment, the kidney, liver, lung and heart organs were immediately obtained after dissecting the Wistar rats, the organs obtained were weighed and transferred into 10% formalin for preservation.

Haematological analysis

Red blood cell (RBC) count was determined by the method described by Miller and Sewaed [17], White blood cell (WBC) count, Haemoglobin determination and Packed cell volume (PCV) were determined by the method described by Snedecor., *et al* [18]. Platelet count were determined by the method described by Miller and Sewaed [17] while Total protein and Albumin were determined by the method described by Doumas., *et al* [19].

Enzyme assays

Alanine Amino Transferase (ALT), Aspartate Amino Transferase (AST) and Alkaline Phosphatase (ALP) were determined by the method described by Reitman and Frankel [20] using a commercially available Kit, GPT-Test Randox (Randox Laboratries Ltd, Anthrim, UK). The assay mixture contained 0.1 mL serum and 0.5 mL of ALT, AST and ALP substrate separately followed by incubation at 37°C for 30 minutes. To the mixture was added 0.5 ml colour reagent (solution 2) and was allowed to stand for 20 minutes at room temperature. Then 5.0 mL of 0.4M sodium hydroxide was added to the mixture. The blank was prepared the same way as the test sample except that 0.1 mL distilled water was added instead of serum. The absorbance was read against the blank after 5 minutes at 546 wavelength. The activity of the enzyme was extrapolated from a calibration curve obtained from an absorbance-enzyme activity table of values provided by the manufactures in the Kit. Enzyme activity was expressed in U/L.

Determination of immunomodulating parameters in Wistar rat

Enzyme linked immunosorbent assay (ELISA) was set up as previously described by Wong., *et al* [21]. Wistar rat serum (0.1 mL) was diluted with phosphate buffered saline (10 mM phosphate pH 7.4, 0.14M NaCl, 0.190 azide) to a final concentration of 10 µg/mL. After the incubation of antibody with standard serum, 100 µl of the mixture was pipetted into each well of the coated plate which had been washed previously three times with phosphate buffered saline containing 0.05% Tween-20 (PBS-Tween). The plates were then incubated for 1h. at room temperature. After incubation, the plates were again washed three times with PBS-Tween buffer and 100 µl of 1/1000 dilution of peroxidase. Avidin-Biotin-peroxide complex (ABC) of 0.1 mL was added and unbound conjugates were washed away with washing buffer. Chromogenic substrate Tetremethyl-1-benzidine (TMB) of 90 µl, a colour developing agent was added into each well and incubated at 37°C in the dark for 15 - 20 minutes. The plates were read using a microplate reader within 30 minutes after adding 0.1 mL of a stop solution at 450 nm.

Histological analysis (Tissues preparation)

The internal organs were exposed by dissection; the kidney and liver were observed for gross lesions. Gross processing involved the description of specimen by their sizes, shapes, colours and overall general appearance. Small portions of each organ already stored in 10% formalin (10% formalin begins the fixation process and prevent autolysis and decomposition) were fixed and put through timed series of dehydration in graded concentrations of xylene. Once the tissues were fixed, they were subjected to several changes of graduated alcohols in a gradient starting at 70% and ending at 100%, a process that removed water from the tissues at a slow controlled rate designed to prevent excessive shrinkage and disruption of the architecture and cellular components. When complete dehydration of the tissues had been accomplished clearing agent was used to remove the alcohol and paraffin. Xylene was used for this purpose. In the next step of processing, heated paraffin infiltrated into the tissues. Hence, the processing involved dehydration, clearing and infiltration of tissues with paraffin wax. The organs were embedded in wax sectioned at 5 micron and transferred on a clean slide. The thin sections were stained with haematoxylin and eosin (H and E) dye for examination under the light microscope for histological study to evaluate structural alterations of organs [22].

Staining technique

The tissue sections were dried completely in an oven because water left on or under the tissue sections can allow the sections to fall off of the slide during the staining process. The slides were then deparaffinized by soaking in xylene, the xylene was removed by alcohol, and the slides were dehydrated by 95% alcohol and then water before staining with haematoxylin. Excess haematoxylin was "blued" by immersion in a weak ammonia water solution. The slides were rinsed again and placed in 80% alcohol and stained with eosin. Excess eosin was removed by alcohol rinses and the slides were prepared for mounting with cover-slips and resinous medium by removal of the alcohol using xylene rinses [23].

Results

Survival of EPS+ producing LAB at varying pH is shown in table 1. *Lactobacillus plantarum* C3, *L. pentosus* G4 and *L. plantarum* Y2 survived at pH 2.0 with colony forming unit of 3 x 10^5, 2 x 10^5, and 4 x 10^5 respectively while *Lc. lactis* G6 and *E. durans* G8 did not survive this condition. However, all the tested LAB survived very well with colony forming unit ranges from 1.1 x 10^8 to 6.4 x 10^9 at pH 3.0 to 6.0.

Lactic Acid Bacteria	pH/viable count (cfu/ml)				
	2.0	3.0	4.0	5.0	6.0
L. plantarum C3	3 x 10^5	1.1 x 10^8	1.9 x 10^9	5.2 x 10^9	3.7 x 10^9
L. pentosus G4	2 x 10^5	1.6 x 10^8	3.6 x 10^9	6.5 x 10^9	4.5 x 10^9
Lc. lactis G6	Nil	3 x 10^8	1.3 x 10^9	3.9 x 10^9	2.8 x 10^9
E. durans G8	Nil	2 x 10^8	2.4 x 10^9	5.7 x 10^9	4.6 x 10^9
L. plantarum Y2	4 x 10^5	1.9 x 10^8	3.1 x 10^9	7.5 x 10^9	6.4 x 10^9

Table 1: *Survival of EPS+ producing LAB at varying pH.*

Key*: C3: L. plantarum; G4: L. pentosus; Y2: L. plantarum; G6: Lc. Lactis; G8: E. durans.*

The comparative effect of bile salt on the growth of LAB isolates is shown in figure 1. In the presence of bile after 4 hours, *L. plantarum* C3 had an optical density of 0.390 ± 0.010 while *L. pentosus* G4 had an optical density of 0.504 ± 0.011. *Lc. lactis* G6, *E. durans* G8 and *L. plantarum* Y2 had optical density of 0.296 ± 0.0051, 0.307 ± 0.013 and 0.540 ± 0.501 respectively as compared to *L. plantarum* C3, L. pen-

tosus G4, *L. lactis* G6, *E. durans* G8 and *L. plantarum* Y2 which had optical-density of 0.475 ± 0.017, 0.603 ± 0.017, 0.364 ± 0.020, 0.415 ± 0.013 and 0.647 ± 0.006 respectively in the absence of bile within the same time interval.

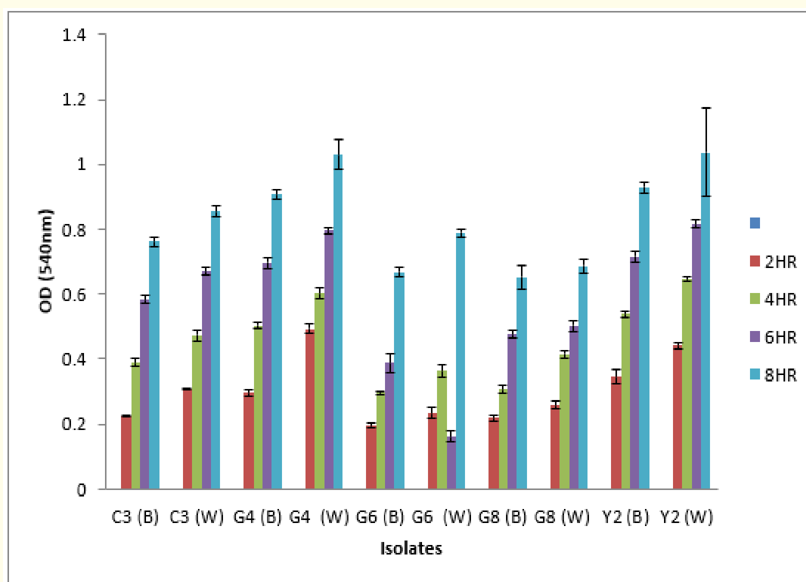

Figure 1: *Influence of bile salt on the growth of LAB.*

The results are mean ± standard deviation of triplicates p < 0.05. The bars on the histogram represent the standard error of means.

Key: C3: L. plantarum; G4: L. pentosus; Y2: L. plantarum; G6: Lc. Lactis; G8: E. durans; (B): Bile Salt; (W): Without Bile.

The antibiotic of last resort for treating gram positive infection is vancomycin (30 μg). In this study, all the isolates were susceptible to vancomycin and were unable to lyse the human red blood cells and none of the isolates was able to liquefy gelatin (Table 2).

Lactic Acid Bacteria	Safety parameters		
	Vancomycin (30 μg)	Heamolytic activity	Gelatinase hydrolysis
L. plantarum C3	S	γ	-ve
L. pentosus G4	S	γ	-ve
Lc. lactis G6	S	γ	-ve
E. durans G8	S	γ	-ve
L. plantarum Y2	S	γ	-ve

Table 2: *Safety assessment of EPS producing LAB.*

Key: *γ (gamma): No haemolysis; -ve: Negative; S: Sensitive.*

The effect of orally administered EPS$^+$ LAB (*L. pentosus* G4 and *L. plantarum* Y2) on biochemical variables in Wistar rats are shown in table 3 to 5. Oral administration with *L. pentosus* G4 and *L. plantarum* Y2 did not significantly (P > 0.05) affect total protein and albumin of Wistar rats.

The mean values of the haemonograms: The Red Blood Cell (RBC), White Blood Cell (WBC), Packed Cell Volume (PCV), Platelet (PLT) and Haemoglobin (HGB) were not significant (P > 0.05) between all the groups (Table 3).

Differential leukocyte counts of the Wistar rats orally administered with EPS producing LAB is shown in table 4. Wistar rats orally administered with *L. plantarum* Y2 (group B) G4 had higher values of lymphocyte (61.00 ± 3.54) while Wistar rats orally administered with *L. pentosus* G4 (11.33 ± 4.02) had higher values of monocyte compared to control group.

Haemogram	Wistar rat group		
	A	B	C
TP	7.30 ± 1.25	7.77 ± 1.63	6.90 ± 1.18
ALB	3.57 ± 0.73	3.86 ± 0.88	3.75 ± 1.02
RBC	5.73 ± 1.14	5.77 ± 1.36	4.63 ± 1.62
WBC	3.14 ± 0.94	3.73 ± 1.6	3.38 ± 1.45
PCV	48.00 ± 10.88	49.00 ± 7.53	34.50 ± 7.44
PLT	313.50 ± 40.31	319.00 ± 19.30	264.00 ± 39.51
HGB	13.87 ± 2.20	15.16 ± 3.58	12.80 ± 2.43

Table 3: *Haemogram of the Wistar rats orally administered with EPS producing LAB.*

Key: **: $p < 0.05$;**: $p < 0.01$; ***: $p < 0.001$. A: Wistar rat orally administered with L. pentosus; B: Wistar rat orally administered with L. plantarum; C: Wistar rat without LAB control; values are Mean ± SD for three replicate analysed. RBC: Red blood cell x 10^2; WBC: White blood cell x 10^9; PCV: Packed Cell Volume (%); PLT: Platelet x 10^3; HGB: Haemoglobin (g/dL); TP: Total Protein (g/dL); ALB: Albumin (g/dL).*

Total and differential leukocytic count	Wistar rat group		
	A	B	C
N	21.50 ± 4.05	24.50 ± 9.19	24.50 ± 1.19
L	54.50 ± 10.61	61.00 ± 3.54	49.00 ± 1.41
M	11.33 ± 4.02	9.50 ± 2.12	8.25 ± 0.96
E	3.00 ± 1.83	3.33 ± 1.52	3.00 ± 0.73
B	2.00 ± 0.08	1.75 ± 0.12	1.67 ± 0.58

Table 4: *Differential leukocytic count of the Wistar rats orally administered with EPS producing LAB.*

Key*: *: $p < 0.05$;**: $p < 0.01$;***: $p < 0.001$ A: Wistar rat orally administered with L. pentosus; B: Wistar rat orally administered with L. plantarum; C: Wistar rat without LAB control; values are Mean ± SD for three replicate analysed. N: Neutrophil; L: Lymphocyte; M: Monocyte; E: Eosinophil; B: Basophil.*

The concentration of serum enzymes in Wistar rats orally administered with *L. pentosus* G4 and *L. plantarum* Y2 shows that alkaline phosphatase (ALP) was high for control group (138.36 ± 19.95 IU/L) compared to the treated groups. However, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were not significantly ($P > 0.05$) different when compared with control group (Table 5).

Enzyme (IU/L)	Wistar rat group		
	A	B	C
AST	55.67 ± 8.62	51.67 ± 4.04	62.86 ± 2.42
ALT	26.67 ± 3.21	32.33 ± 2.89	34.89 ± 9.75
ALP	126.00 ± 12.73	122.50 ± 9.19	138.36 ± 19.95

Table 5: *Enzymatic analyses of the Wistar rats orally administered with EPS producing LAB.*

Key: **: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Wistar rat orally administered with L. pentosus; B: Wistar rat orally administered with L. plantarum; C: Wistar rat without LAB control; values are Mean ± SD for three replicate analysed. AST: Aspartate amino transferase; ALT: Alanine Amino Transferase; ALP: Alkaline Phosphatase.*

All groups did not show significant differences (P > 0.05) in the levels of gamma interferon (IFN-γ), tumour necrotic factor alpha (TNF-α) and immunoglobulin G (IgG) (Table 6). However, the `Wistar rats orally administered with *L. pentosus* G4 and *L. plantarum* Y2 had increase values of immunoglobulin A (IgA) and interleukin-2 (IL-2) compared to the control groups. However, group B (rats orally administered with *L. plantarum* Y2 showed high significant (P < 0.01) difference in the levels of IgA and IL-2 (1205.47 ± 15.09 ng/ML and 30000.00 ± 33.29 pg/mL).

Immunological parameter	Wistar rat group		
	A	**B**	**C**
TNF-α (pg/ML)	1056.90 ± 140.82	1138.21 ± 186.29	1300.81 ± 140.82
IFN-γ (pg/ML)	1375.66 ± 183.29	2921.05 ± 873.85	1375 ± 91.64
IL-2 (pg/ML)	2491.22 ± 76.49	3000.00 ± 33.29**	1492.06 ± 86.32
IgA (ng/ML)	973.20 ± 18.18	1205.47 ± 15.09**	738.49 ± 24.39
IgG (ng/ML)	688.93 ± 34.00	694.53 ± 25.96	675.10 ± 26.16

***Table 6:** Immunological analyses of the Wistar rats orally administered with EPS producing LAB.*

Key:** *: p < 0.05;**: p < 0.01;: p < 0.001. A: Wistar rat orally administered with L pentosus; B: Wistar rat orally administered with L plantarum; C: Wistar rat without LAB control; values are Mean ± SD for three replicate analysed. TNF-α: Tumor Necrotic Factor Alpha; IFN-γ: Interferon Gamma; IL-2: Interleukin-2; IgG: Immunoglobulin G; IgA: Immunoglobulin A.*

The weights (sizes) of the internal organs such as heart, lung, liver and kidney revealed no significant difference but a significant elevation of lung weight (0.36 ± 0.04g) was observed in group B (Wistar rats orally administered with *L. plantarum* Y2) compared to group A and control group as shown on table 7. The experimental rats showed increased in body weight though no death occurred. All the rats appeared healthier and active till the time they were sacrificed. No morphological changes such as necrosis, vascular congestion and interstitial infiltration seen on the excised organs.

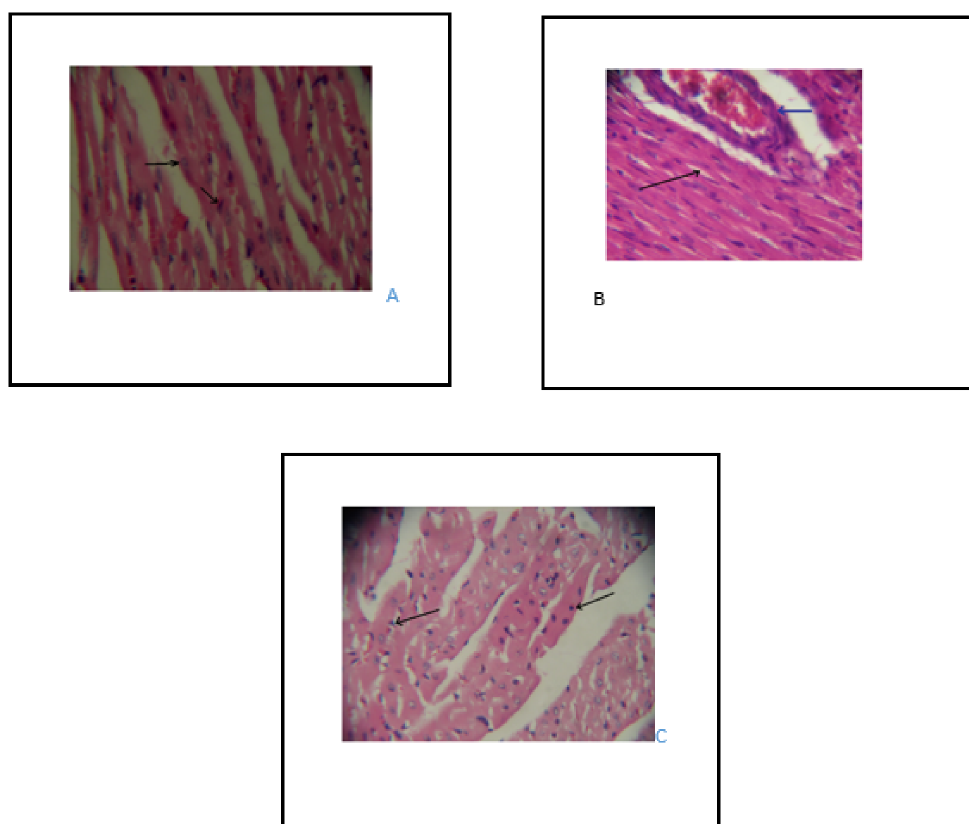

***Plate 1:** Photomicrograph of the heart of Wistar rats orally administered with LAB species.*

Key: A (Wistar rats orally administered with *L. pentosus*), B (Wistar rats orally administered with *L. plantarum*) and C (Control) *(Magnification x 10).*

Black arrow showing normal endocardial layer.

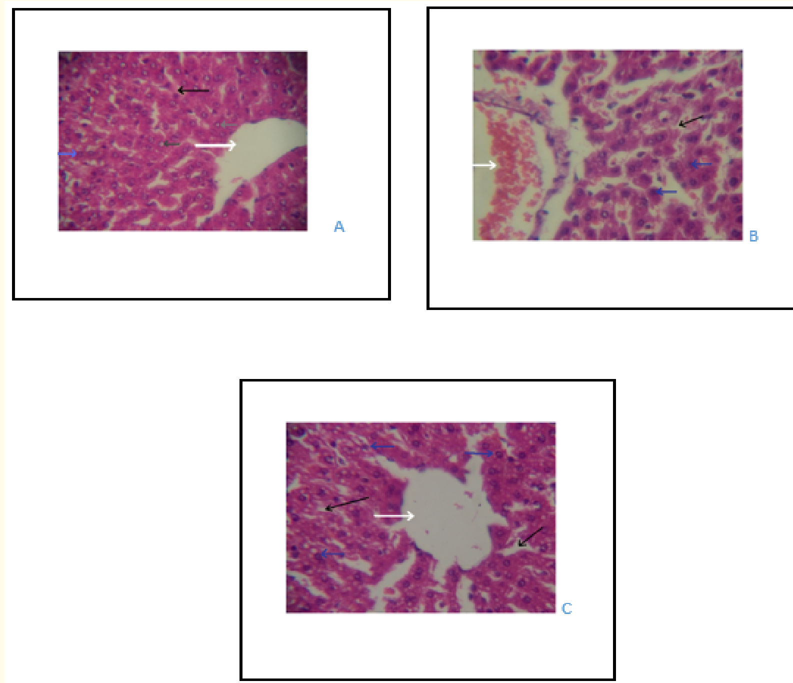

Plate 2: *Photomicrograph of the liver of Wistar rats orally administered with LAB species.*

Key: *A (Wistar rats orally administered with L. pentosus), B (Wistar rats orally administered with L. plantarum) and C (Control) (Magnification x 10).*

Normal vessel (white arrow), Sinusoids (black arrow) and Normal Hepatocytes (blue arrow).

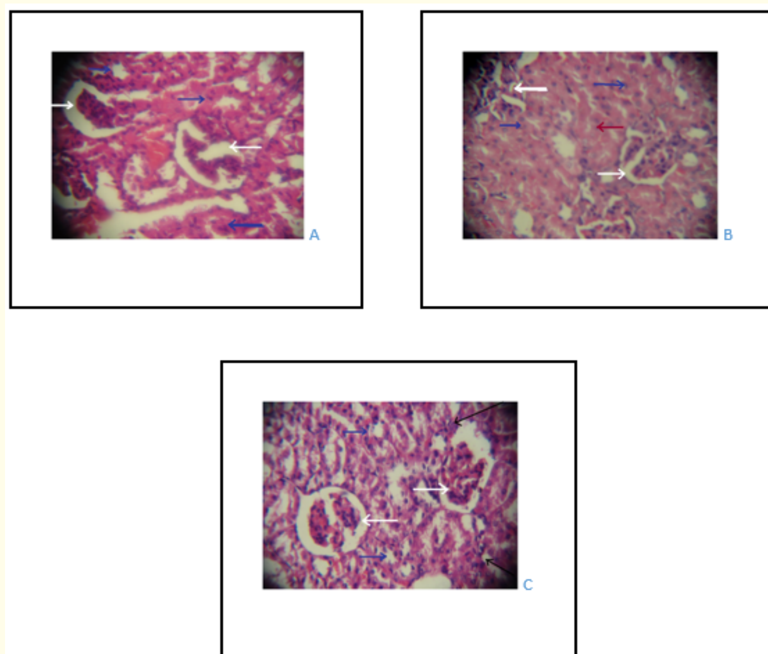

Plate 3: *Photomicrograph of the kidney of Wistar rats orally administered with LAB species.*

Key: *A (Wistar rats orally administered with L. pentosus), B (Wistar rats orally administered with L. plantarum) and C (Control) (Magnification x 10).*

Normal glomeruli (white arrow) and Mesangial cells (black arrow).

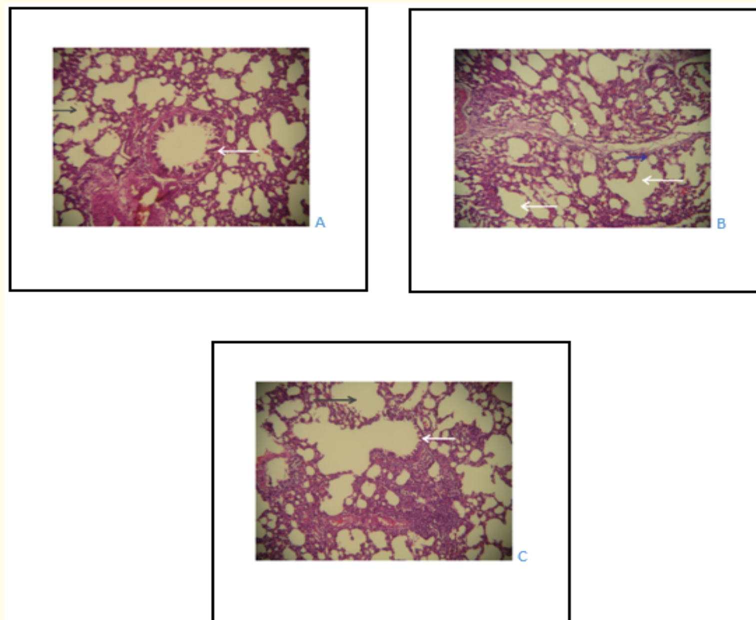

Plate 4: *Photomicrograph of the lungs of Wistar rats orally administered with LAB species.*

Key: *A (Wistar rats orally administered with L. pentosus), B (Wistar rats orally administered with L. plantarum) and C (Control) (Magnification x 10).*

Normal bronchiole (white arrow) and Alveolar duct (black arrow).

Weight/organ (g)	Wistar rat group		
	A	B	C
W1	164.78 ± 7.16	166.77 ± 6.61	163.80 ± 12.91
W2	179.33 ± 8.68	183.67 ± 5.69	166.33 ± 14.22
Kidney	1.33 ± 0.15	1.45 ± 0.14	1.30 ± 0.14
Liver	5.11 ± 0.26	5.06 ± 0.29	4.95 ± 0.41
Lung	0.29 ± 0.03	0.36 ± 0.04*	0.26 ± 0.02
Heart	0.47 ± 0.03	0.48 ± 0.06	0.45 ± 0.04

Table 7: *Weight of the Wistar rats and weight of vital organs excised from Wister rats.*

Key: **: $p < 0.05$;**: $p < 0.01$;***: $p < 0.001$. A: Wistar rat orally administered with L pentosus; B: Wistar rat orally administered with L plantarum; C: Wistar rat without LAB control; values are Mean ± SD for three replicate analysed; W1: Initial Body Weight of Wistar rat; W2: Final Body Weight of Wistar rat.*

Wistar rat group	Identified part	Comment
A	Kidney	Appears normal
	Liver	Appears normal
	Lung	Appears normal
	Heart	Appears normal
B	Kidney	Appears normal
	Liver	Appears normal
	Lung	Appears normal
	Heart	Appears normal
Control	Kidney	Appears normal
	Liver	Appears normal
	Lung	Appears normal
	Heart	Appears normal

Table 8: *Histopathology examination of the Wistar rat orally administered EPS+ producing LAB.*

Key: *A: Wistar rat orally administered with L. pentosus; B: Wistar rat orally administered with L. plantarum; C: Wistar rat without LAB control.*

Discussion

Species of EPS+ LAB isolated in this work include *L. plantarum* C3, *L. pentosus* G4, *Lc. lactis* G6, *E. durans* G8 and *L. plantarum* Y2 and the tests organisms were able to survived acidic conditions at various pH in agreement with the report of Ogunbanwo [24]. This indicated that the LAB strains were able to survive under the pH conditions usually found inside the gastrointestinal tract as previously reported [25].

According to EFSA [26], there is a strong need for valid safety assessments prior to commercial use of LAB. In this study, the LAB was susceptible to vancomycin, hence can be used as probiotic. Indiscriminate use of antibiotic and presence of resistance genes are responsible for antibiotic resistance development by bacteria.

LAB used in this work exhibited gamma haemolysis. They were unable to lyse the human red blood cells and none of the isolates was able to liquefy gelatin. The investigation of the virulence factors in EPS+ LAB is of great importance to determine the absence of pathogenicity traits such as gelatinase and haemolytic activity [27]. Gelatinase is an extracellular protease involved in the hydrolysis of gelatine, casein, collagen, haemoglobin and other bioactive proteins [28]. Haemolytic activity is considered as an important virulence factor and a good indicator in order to select potential probiotic strains that do not have such trait.

In this study, *L. pentosus* G4 and *L. plantarum* Y2 reinforced the host defence systems by secretion of the polymeric IgA. Perdigon., *et al*. [29] reported that orally administered LAB (*L. acidophilus* and *L. casei*) and yoghurt feeding increased secretory immunoglobulin A (IgA) production and the number of IgA-producing cells in the small intestine of mice in a dose-dependent manner. These investigators proposed that IgA secreted by the intestinal B cell enters the circulations and raises the serum IgA concentration. In addition, *L. plantarum* Y2 induced significantly secretion of IL-2 and TNF-α, this is known to shift the immune system towards a T helper 1 (Th 1) type of response.

Abnormal toxicity studies did not show any apparent changes in the behaviour of Wistar rats. orally administered with *L. pentosus* G4 and *L. plantarum* Y2. In addition, Wistar rats orally administered with LAB survived throughout the experiment. Haematological parameter shows that the haemoglobin fell within the normal limit. However, animal's defensive mechanisms can react quite differently to different bacteria; therefore, there is no singular pattern in heamonogram that indicated a bacterial infection. The similarity in values for packed cell volume, Hemoglobin and red blood cell count of control indicated that the animals were not susceptible to anemia related diseases and that the treatments seemed to be capable of supporting high oxygen carrying capacity in the experimental animals [30]. Similarity in white blood cell of Wistar rats administer with *L. pentosus* G4 and *L. plantarum* Y2 and the control is an indication that the animals were not fighting against any disease condition.

Safety assessments of *L. pentosus* G4 and *L. plantarum* Y2 were carried out using serum enzymes (AST, ALT and ALP), total protein and albumin. The toxicological assessments results revealed that there was no significant increase in the level of all the biomarkers and fell within normal value. In this study, the liver and kidney from the Wistar rats orally administered with *L. pentosus* G4 and *L. plantarum* Y2 were similar to those from the control. The liver is the main organ in the detoxification and metabolism of chemicals, and AST, ALT and ALP are important liver enzymes that can be used as good indicators for liver function because any damage in the hepatic cells will results in an increase in the serum levels of these enzymes Chemistry [31]. According to the American Association for Clinical Chemistry [32], low levels of albumin and total protein are good indicators for problematic functions of both liver and kidney. Also, low level of total protein may occur in inflammatory bowel disease.

The above interpretation of the haematological analysis is only suggestive; however, histopathological analysis gave more concrete and reliable explanations. The Wistar rats orally administered with *L. pentosus* G4 and *L. plantarum* Y2 exhibited no tissue disruption. There was no congestion, hemorrhage nor necrosis seen in both the treated and control Wistar rats. The increase in the size of the lung of rat orally administered with *L. plantarum* Y2 may have been due to an increase in body weight of the albino rat. Blood sample revealed normal serum enzymes, normal protein, albumin and heamogram. Serology findings are consistent with protective immunity displayed by LAB.

Conclusion and Recommendation

In conclusion, fermented dairy products are rich sources of LAB especially *Lactobacilli* which were characterized and identified. *Lactobacillus plantarum* Y2 stimulated immunomodulatory activities at accepted safety levels and may justify its uses as an immunomodulator. Hence, can be used to complement synthetic antibiotics as it helps to stimulate immune response to suppress infections of the Wistar rats.

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