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Research article

In vitro antioxidant, antibacterial, in vivo immunomodulatory, antitumor and hematological potential of exopolysaccharide produced by wild type and mutant *Lactobacillus delbureckii* subsp. *bulgaricus*

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ABSTRACT

Biological evaluation of exopolysaccharides (EPS) produced by wild type and mutant *Lactobacillus delbureckii* (EPSWLD and EPSMLD) was investigated. Varying degrees of functional groups associated with polysaccharides were present thus confirming the EPS. The EPSs had strong antioxidant potential in a dose dependent (0.5–10 mg/mL) manner. EPSWLD and EPSMLD exhibited the highest 1,1-diphemy 1-2-picryl-hydrazyl (DPPH) activity (73.4 % and 65.6 %), total antioxidant activity (1.80 % and 1.42 %), H_2O_2 scavenging activity (88.5 % and 78.6 %) and Ferric Reducing Antioxidant Power (FRAP) (1.89 % and1.81 %) at 10 mg/mL respectively. WLD and MLD were highly susceptible to chloramphenicol, cotrimoxazole, tetracycline, erythromycin and ceftazidine and resistant to cefuroxime, gentamicin and cloxacillin. The EPSs had antibacterial activity against the test pathogens. *B. subtilis* and *S. aureus* had the highest susceptibility (26.0 mm and 23.0 mm). EPSMLD modulate the highest IgG, IgA and IgM production (68–126 mg/dL and 67–98 mg/dL and 64–97 mg/dL) in the treated tumor induced mice (TTIM). EPSWLD and EPSMLD exhibited reduction capability on the CEA level (3.99–4.35 ng/L and 4.12–4.23 ng/L) of the TTIM. EPSWLD TTIM had the highest amount of RBC, WBC and PCV (5.6×10^{12}%, 68000% and 42%). The EPS increased the lifespan of TTIM. In conclusion EPSWLD and EPSMLD had strong biological potential with pharmacological and neutraceutical activity.

1. Introduction

Lactobacillus delbrueckii subsp *bulgaricus* is a nonpathogenic microorganism that produces lactic acid which is largely used in dairy industries, especially in cheese-making and yoghurt production. It has the ability of changing the intestinal milieu, reduces lactose intolerance and also improves the immune system (Piard and Desmazeaud, 1992). Strains of Lactic Acid Bacteria (LAB) such as *Streptococcus, Lactococcus, Pediococcus, Lactobacillus, Leuconostoc* and *Weissella* species are frequently known to produce (EPSs (Patel and Prajapati, 2013; Adebayo-Tayo et al., 2018). EPSs that are naturally produced are highly susceptible to biodegradation and are less harmful than synthetic polymers (Boudjek et al., 2015; Wang et al., 2014; Ngan et al., 2014).

Microbial EPSs refers to all forms of bacterial polysaccharide, both slime and capsule, found outside the cell wall (Prathima et al., 2001). LABs are able to produce EPSs in the surrounding medium as slime or on the surface of bacterial cells to form a capsule (Ramchandran and Shah, 2009). EPSs producing LAB such as *Streptococcus thermophilus, L. delbrueckii subsp. bulgaricus, Lactococcuslactis subsp. cremoris* isolated from dairy products and fermented milk have been extensively studied (Patel and Prajapati, 2013) and may provide physiological benefits which include antioxidant activities, antitumor, immunomodulation and cholesterol lowering ability (Zhang et al., 2013a, 2013b; Li et al., 2014; Shao et al., 2014; Adebayo-Tayo et al., 2018).

Free radicals are harmful to living organisms (Mahapatra and Banerjee, 2013). To reduce the damage caused by free radicals, both synthetic and natural antioxidants are used. Butylated hydroxyanisole (BHA), butylated hydroxyto-luene (BHT) and n-propyl gallate (PG) are examples of synthetic antioxidants with potent antioxidant activity against several oxidation systems which results in carcinogenesis and liver damage thereby posing potential risks in human system (Luo and Fang, 2008; Liu et al., 2009). This results in the development of natural nontoxic antioxidants to protect humans from free radicals. Exploitation of safe natural antioxidants from bio-resources such as exopolysaccharides that can replace synthetic antioxidants has gained great importance in science and medicine because of their ability to maintain human health as well as

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treatment of diseases (Li, 2012). LAB exhibit antioxidant activity in four major ways; they may reinforce the inherent cellular antioxidant defense by secreting enzymes like superoxide dismutase (SOD). They also release and promote the production of the major non-enzymatic antioxidant and free radical scavenger glutathione (GSH). Moreover, they promote the production of certain antioxidant biomolecules, such as the exopolysaccharides (EPSs). Finally, they exhibit metal chelating activity. Superoxide anion and hydrogen peroxide degradability potential and reduction of Rreactive Oxygen Species (ROS) accumulation risk through indigestion of food by LAB has been reported (Liu and Pan, 2010).

Antimicrobials have been used increasingly as a primary intervention for inhibition or inactivation of pathogenic microorganisms in foods (Davidson and Zivanovic, 2003). EPSs from LAB also produces antimicrobial compounds including hydrogen peroxide, CO_2, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins (Cintas et al., 2001). Generally, food antimicrobial agents are not used alone to control foodborne pathogens, but are included as components of the multiple approaches to microbial control.

EPS from LAB are good candidates for immunotherapeutic agent against cancer because they usually have low side effect and are less cytotoxic (Yu et al., 2009; Osuntoki and Korie, 2010). EPS from *Lactobacillus delbrueckii* strains can enhance humoral immunity mediated by immunoglobulins produced by the bone marrow lymphocytes (B lymphocytes). The B lymphocytes are responsible for specific recognition and removal of antigens that are extracellular located. The EPS from LAB that can also enhance cell-mediated immune responses such as natural killer cell tumoricidal activity, T-lymphocyte proliferation and mononuclear cell phagocytic capacity has been reported (LeBlanc et al., 2002). This research work is therefore aimed at determining the antioxidant, immunomodulatory, antitumor and hematological potential of exopolysaccharide produced by wild and mutant *Lactobacillus delbureckii* subsp *bulgaricus*.

2. Materials and methods

2.1. Culture collection

The wild and mutant *Lactobacillus delbrueckii* and the test pathogens (*Bacillus subtilis, S. typhi*, *E. coli*, *K. pneumonia, S. dysentriae* and *S. aureus*) used in this experiment were collected from the culture collection of our previous work in Microbial Physiology Unit, Department of Microbiology, University of Ibadan (Ishola and Adebayo-Tayo, 2018). The isolates was maintained in De Man, Rogosa and Sharpe (MRS) broth (De Man et al., 1960) and stored at 28 °C for three days.

2.2. Production, extraction and quantification of EPSWLD and EPSMLD

Exopolysaccarides was produced using wild and mutant *Lactobacillus delbrueckii* using Modified Exopolysaccharide Selection Medium (De Man et al., 1960). The inoculated medium was incubated at 30 °C for 16 h (Adebayo-Tayo and Onilude, 2008). The supernatant from the fermentation medium was precipitated at 4 °C by the addition of 2 volume of ethanol (100%). Total sugar of the precipitate was determined using phenol-sulphuric acid method (Dubois et al., 1956). The amount of sugar present and their levels were calculated using glucose as standard.

2.3. Characterization of the EPSWLD and EPSMLD

2.3.1. Fourier Transform Infrared (FTIR) analysis of the EPSWLD and EPSMLD samples

The major structural groups of the purified EPS were detected using Fourier Transform Infrared (FT-IR) spectroscopy. The spectrum of the EPS was obtained using a KBr method. The polysaccharide samples were pressed into KBr pellets at a sample ratio of 1:100. The FT-IR spectra were recorded on a Bruker Tensor 27 instrument with a resolution of 4 cm^{-1} in the region of 4, 000–400 cm^{-1} (Fontana et al., 2015).

2.3.2. Determination of the monosaccharide composition of the EPSWLD and EPSMLD using high performance liquid chromatography

The monosaccharide composition of EPSWLD and EPSMLD was determined using HPLC (Honda et al., 1989; Yang et al., 2012). The rehydrated EPSWLD and EPSMLD samples was hydrolyzed uing 1 mL of 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h, derivatized with 1-phenyl-3-methyl-5-pyrazolone and subsequently analyzed by high-performance liquid chromatography (HPLC) with a four-unit pump (Agilent Technologies, Wilmington, USA) and a Shim-pak VPODS column (4.6 × 150 mm) with detection by absorbance monitoring at 245 nm. The mobile phase consisted of 82% sodium phosphate (50 mM, pH 7.0) and 18% acetonitrile (v/v), and the sample was eluted at a flow rate of 1.0 mL/min with the use of refractive index detector. An integrator was used to measure the area under the curves and by comparing different sugar standard with a known retention time of the EPS sample.

2.4. Determination of antibiotic susceptibility of WLD and MLD

The susceptibility of the isolates to different types of antibiotics was done using Agar Disc Diffusion method (Bauer et al., 1966). Commercially available antibiotics disc (Oxoid) containing ceftazidine (30 μg), cefuroxine (30 μg), erythromycin (5 μg), gentamycine (10 μg), cefixime (5 μg), ofloxacin (5 μg), augmentin (30 μg), chloramphenicol (30 μg), tetracycline (10 μg), ciprofloxacine (5 μg), amoxycillin (25 μg), cotrimoxazole (25 μg), nitrofurantion (300 μg) and cloxacillin (5μg) were used. Young culture of WLD and MLD were aseptically swab on MRS agar plates and the antibiotics disc were placed on the surface of the agar plates. The inoculated plates were incubated at 37 °C for 24 h and zone of inhibition were measured and recorded in diameter (Vlkova et al., 2006).

2.5. Antibacterial potential of the EPSWLD and EPSMLD

The antimicrobial potential of the EPSWLD and EPSMLD was determined using Agar Well Diffusion method (Honda et al., 1989; Sarrazin et al., 2012) using *Bacillus subtilis, S. typhi*, *E. coli*, *K. pneumonia, S. dysentriae* and *S. aureus* as test pathogens. The suspension of the tested microorganism (1×10^6 CFU/ml) was seeded on Mueller - Hinton Agar (Lab M Ltd., UK) plates. Uniform wells were cut on the dried agar plate using a sterile cork borer of diameter 7 mm. Each well was filled with 10 μL of the EPSWLD and EPSMLD. The inoculated plates were incubated at 37 °C for 24 h. After incubation the plates were observed for zones of inhibition (ZOI) around the wells and the ZOI diameters (mm) were recorded.

2.6. Determination of the antioxidant potential of the EPSWLD and EPSMLD

The free radical scavenging activity of the exopolysaccharide was measured by 1-1-diphenyl-2-picrylhydrazyl (DPPH) radicals according to Shimada et al. (1992). Five milliliters of 0.1 of DPPH ethanol solution (freshly prepared) were added to different concentration of EPS (50, 100, 250, 500 and 1000 μL). The reaction was kept in the dark for 30 min. Absorbance was measured at 517 nm using Spectrophotometer UV-visible 2401PC (Shimadzu, Japan). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The experiment was carried out in triplicate and averaged (inhibition percentage plotted against concentration of tested polysaccharide). The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging activity } (\%) = [1-(A_{sample}-A_{blank})/A_{control}] \times 100$$

Hydrogen Peroxide scavenging potential was done following the method of Thampi and Shalini (2015). 0.1M Phosphate buffer (pH 7.4) was prepared in 10mM solution of Hydrogen Peroxide.1mL of different concentration of EPS (50, 100, 250 and 1000 mg/mL) was added to 2 mL of the Hydrogen Peroxide solution. Absorbance of the reaction mixture

was taken at 230nm after 10 min of incubation at 30 °C against blank solution using UV- spectrophotometer. Ascorbic acid was used as standard.

$$\text{Scavenging activity } (\%) = (A_{sample}–A_{blank})/A_{control} \times 100$$

Total Antioxidant Activity of the EPS samples was determined (Dilna et al., 2015). Total antioxidant capacity of the EPS samples was determined by mixing 7.45mL Sulfuric acid (0.6M), 1.235g of Ammonium Molybdate (4mM), 0.9942g of Sodium Sulphate (28mM) in 250mL distilled water (Total antioxidant capacity reagent). 0.1 mL of different concentration of EPS (50,100, 250, 500 and 1000 mg/mL) was dissolved in 1 mL of total antioxidant capacity and absorbance was taken at 695 nm after 15 min. Ascorbic acid was used as standard.

The reducing power of the EPS was evaluated using the method modified by Gülçin et al. (2010). 2.5 mL of 2 mM Phosphate buffer (pH 6.6), 1% of Potassium Ferricyanide (2.5 mL) and 1 mL distilled water was mixed with different concentration of EPS (200, 400, 600, 800 and 1000 mg/mL). The mixture was kept for 20 min at 50 °C. 2.5mL of 10% Trichloroacetic acid was added to the reaction and spinned at 3000 rpm for 10 min. 1% ferric chloride (0.1mL) and 2.5 mL distilled water was added. The reaction mixture was allowed to stay for 10 min after which the absorbance was taken at 700 nm. Ascorbic acid was used as standard.

2.7. Determination of antitumor potential of the EPSWLD and EPSMLD

2.7.1. Rearing condition of the animals and exposure method and experimental design

All procedures used for the animal study conforms to the guidelines for Animal Care Use and Research Ethics Committee (ACUREC), University of Ibadan. 8–10 weeks old weighing 20–24 g female Swiss albino mice (20) maintained in the Animal Breeding Unit, University of Ibadan was used. The mice were fed with pelletized rat feed and water *ad libitum,* permitted to adapt to the laboratory surroundings for two weeks prior to the experiment.

Smoking of cigarette was done two times in a day for five days for four month in an enclosed compartment. The side stream smoke was created by burning Little London cigarettes in a smoking compartment. The cigarettes were ignited and allowed to flare in the chamber. Whole body of the four experimental groups of mice was subjected to the side stream ignited cigarette.

Mice were randomly grouped into five different groups (five mice in each); Grp 1 (mice that are not exposed to cigarette), Grp 2 (mice exposed to the cigarettes smoke and was not treated); Grp 3 (mice exposed to the smoke of cigarettes and treated with 20 mg/kg/day 5-fluorouracil); Grp 4 (mice exposed to the cigarettes smoke and treated with 500 nM aqueous solution of EPSWLD through intra-peritoneal (IP) injection) and Grp 5: (mice exposed to the cigarettes smoke and treated with 500 nM aqueous solution of EPSMLD through intra-peritoneal (IP) injection). All treatments were administered for 15 days. The antitumor efficiency of EPS was juxtapose with that of 20 mg/kg/day 5-fluorouracil IP for nine days (Muthlu et al., 2009).

2.7.2. Determination of immunoglobulin

The assay for antibodies was based on turbidimetric measurement. Turbidity was caused by the formation of antigen-antibody insoluble immune complexes. For the determination of IgG, IgA and IgM, the following reagents were used: Blood serum, (phosphate buffer saline-pH 7.4), IgG, IgA and IgM Antibody, Sodium azide and distilled water. The IgG, IgA and IGM of the treated and untreated mice were determined by diluting the blood serum samples and the control in 0.9% saline (1:10). 20μL of the diluted samples was added to 900 μL of phosphate buffer and labeled sample A2. The absorbance of sample A1 was taken at 340 μL. 100 μL antibody reagent was added into the prepared samples and mixed properly. The reaction mixture was incubated for 15mins. Absorbance of A2 and control was taken at 340nm.

2.7.3. Determination of hematology analysis of the EPSWLD and EPSMLD

The mice were anaesthetized with chloroform after which blood samples were taken by intracardiac puncture. Blood sample was promptly transferred into ethylenediaminetetra-acetic-coated vials (EDTA) for determination of possible hematological test. Hematological assay (white blood cell (WBC), red blood cell (RBC), platelet level, percentage Packed Cell Volume (PCV)) as well as Measurement of Mean Corpuscular Hemoglobin Concentration (MCHC) of each sample was determined using standard laboratory procedures.

2.7.4. Ichroma^{tm}CEA test

Plasma and serum were separated from the whole blood obtained from the tumor induced mice within 3hrs by centrifugation. 150 μL of plasma and control sample was transferred into a test tube containing the detection buffer and thoroughly shaking for 10 min before dispensing on the test cartridge through its well. The cartridge was allowed to stay at room temperature for 12 min and then inserted into the holder. The cartridge was scanned by the ichroma™ reader.

2.8. Statistical analysis

All experiments were performed in triplicates and the results were statistically subjected to Analysis of Variance (ANOVA) using SPSS (version 11.0, Chicago, IL). Probability values ($P = 0.05$) was considered significant to indicate difference. Duncan's Multiple Range Test ($P = 0.05$) were used to determine the significant difference.

3. Results and discussion

Production of EPS by the wild and mutant *L. delbrueckii*.

Production of EPS by the wild and mutant *L. delbrueckii* is shown in Figure 1. The EPS produced by the wild and mutant *L. delbrueckii* ranged from 5570.34 – 5910.62 mg/L. Wild *L. delbrueckii* (WLD) had the highest amount of EPS.

This result is in line with our previous work in which wild *Lactobacillus delbrueckii* (WLDYG2) had the highest EPS production (Ishola and Adebayo-Tayo, 2018). Glucose and glucose moiety was reported as source of sugar for biosynthesis of heteropolysaccharides (Escalante et al., 1998).

Figure 2a – b showed the infrared spectra of the major functional groups of EPSWLD and EPSMLD. The distribution of functional groups present in EPSWLD and EPSMLD is shown in the Table 1. The EPS showed that peaks at 3404.47 cm^{-1} and 3385.18 cm^{-1} were characteristics of O–H which existed in hydrogen bond of polymer indicating that the compound is likely to be unsaturated or aromatic and a weak peak at 2364.81 cm^{-1} and 2360.95 cm^{-1} indicate an asymmetrical C–H stretching bond. Unsaturation of the EPS was more highlighted at peaks 1637.62 and 1641.48 cm^{-1} and these peaks were assigned to the starching vibration of the carboxyl group (C=O).

The FTIR spectra of EPSWLD and EPSMLD are shown in Figure 2a and b.

Water associated with this peak indicates the presence of organic substances such as the ring stretching of mannose or galactose (Wang

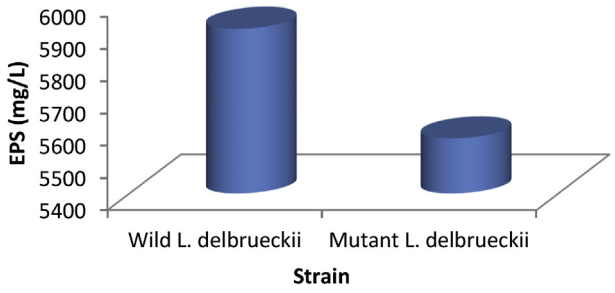

Figure 1. Production of EPS by the wild and mutant *Lactobacillus delbrueckii.*

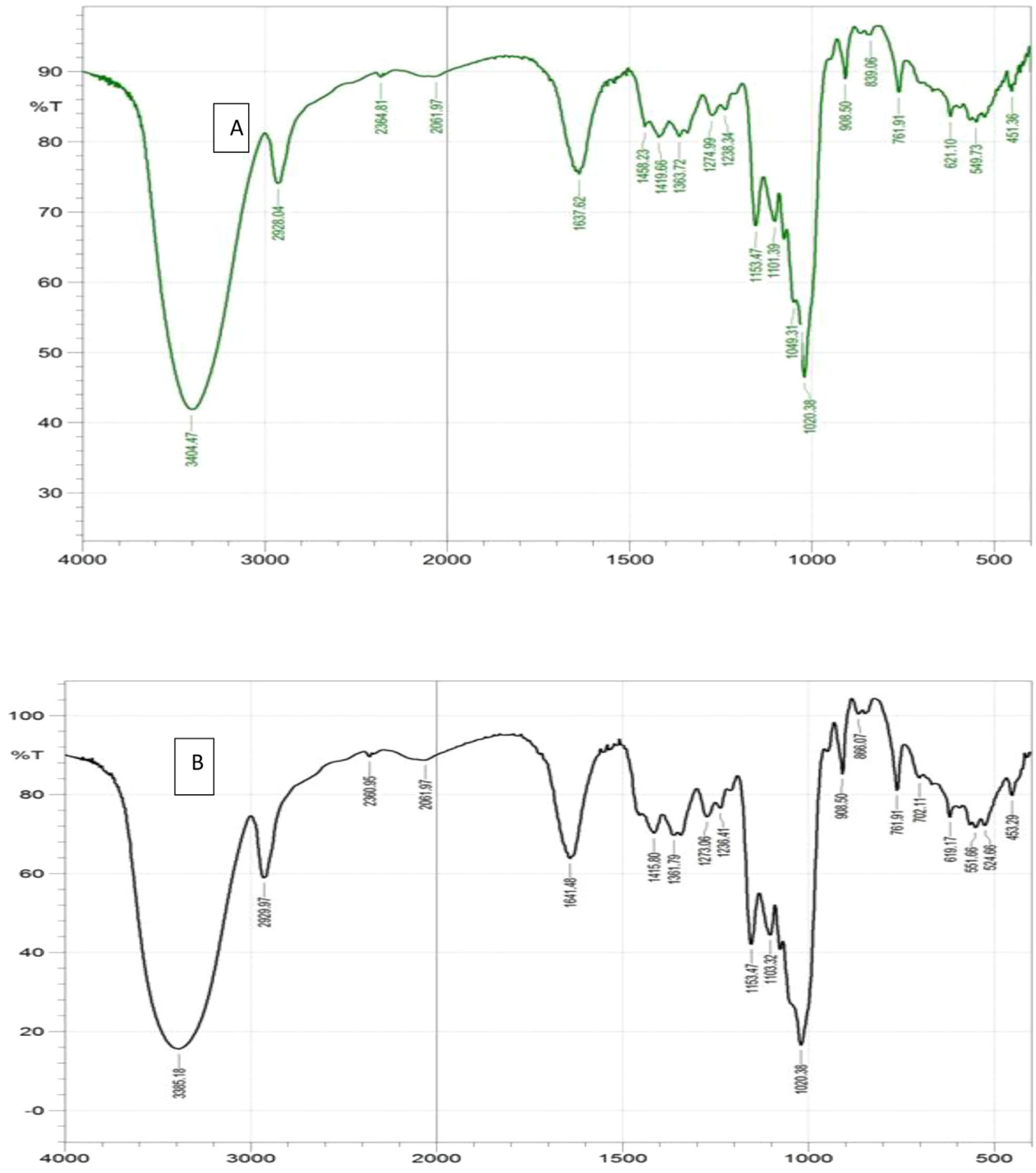

Figure 2. a–b: FTIR spectrum of (a) EPSWLD and (b) EPSMLD.

et al., 2015). The strong absorption peak at 1, 020.38 and 1,049.31 cm^{-1} indicated the presence of carbohydrates (Abdhul et al., 2014). The EPSWLD differ from EPSMLD in three peaks less in the regions 1, 236.41 cm^{-1}; 1,274.99 cm^{-1}; 2,928. 04–2929.97 cm^{-1} which indicates lesser OH presence in the EPSWLD as this region is characteristics of alcohol and aromatic bonds (Adebayo-Tayo et al., 2017, 2018). Absence of peaks in the range of 260–290 cm^{-1} clearly indicated that the sample did not have any proteins and nucleic acids (Bai et al., 2016).

3.1. Monosaccharide composition of the EPS

The retention time, area under curve and sugar concentration of the EPSWLD and EPSMLD samples is shown in Table 2. There was a significant difference ($P \leq 0.05$) in sugar concentration of the EPS produced by EPSWLD and EPSMLD. Sugars such as ribose, xylose, arabinose, rhamnose, fructose, glucose, mannose and galactose were present in the EPS samples produced by both wild and mutant *Lactobacillus delbrueckii.* The

Table 1. Distribution of functional group of the FTIR spectra of EPSWLD and EPSMLD.

S/N	Peak (cm^{-1})	Functional group	EPSWLD	EPSMLD
1	453.29	Chloroalkanes	+	+
2	524.66. 549.73, 551.66	Alkyl halide	+	+
3	619.17, 621.1	Alkynes	+	+
4	702.11, 761.91	Alkanes	+	+
5	839.06, 866.07	Alkyl halide	+	+
6	908.5	Aromatics	+	+
7	1020.38, 1049.31	Alkenes	+	+
8	1101.39, 1103.32	Alkenes	+	+
9	1153.41	Alkenes	+	+
10	1274.99	Alkanes	+	-
11	1236.41	Aliphatic amines	-	+
12	1363.72, 1273.06	Alkanes	+	+
13	1419.66, 1415.8	Ester	+	+
14	1637.62, 1641.48	Un-Saturated Ester	+	+
15	1658	Amide	+	-
16	2061.96	Alcohol	+	+
17	2364.81, 2360.95	Alcohol	+	+
18	2928.04, 2929.97	Aldehyde	+	-
19	3404.47, 3385.18	Hydroxyl/alcohol	+	+

sugar concentration ranged from 2.09938 – 39.8584 mg/100g and 1.48119–23.85038 mg/100g respectively. Glucose had the highest concentration in EPSWLD contained higher glucose compared to EPSMLD which contained higher concentration of galactose. EPSMLD contained less Ribose compared to EPSWLD.

This is in line with the result of Li et al. (2014) that EPS from *L. helveticus* was mainly composed of galactose, glucose, and mannose (Li et al., 2015). The present of eight different sugar moieties present in the EPSWLD and EPSMLD indicates that the EPS was heteropolysaccharide. It is also in line with the work of Dolyeres who reported that galactose and glucose are the dominant sugars.

3.2. Determination of Antioxidant potential of the EPSWLD and EPSMLD

Table 3 shows the different radical scavengers (DPPH, OH-, Total antioxidant capacity and reducing power capacity) used to determine the antioxidant properties of EPSWLD and EPSMLD. The DPPH scavenging activity ranged from 38.5 to 73.4% and 37.5–65.6%, and total antioxidant capacity ranged from 1.21% to 1.80% and 0.41% to 1.42 % for EPSWLD and EPSMLD. H_2O_2 and FRAP ranged from 50.6 % to 88.5 % and 33.1% to 78.6 % and 1.23–1.89% and 1.0 to1.81 %for EPSWLD and EPSMLD. There was a significant difference ($P \leq 0.05$) in the DPPH, OH^-, Total antioxidant capacity and FRAP activity. Generally the antioxidant activity increased with increase in EPS concentration. Both EPSWLD and EPSMLD exhibited higher scavenging activity than ascorbic acid which was used as control.

EPSWLD exhibited the highest H_2O_2 and FRAP scavenging activity compared to EPSMLD. EPSWLD and EPSMLD had higher H_2O_2 and FRAP scavenging potential compared to ascorbic acid. Generally, the highest antioxidant scavenging activity was recorded at 10 mg/mL EPSWLD and EPSMLD concentration. At all concentrations the EPSWLD and EPSMLD showed higher activities than ascorbic acid which was used as the control. The EPSWLD exhibited higher antioxidant capacity than EPSMLD.

Higher antioxidant activity exhibited by the EPSWLD and EPSMLD could be as a result of the bioactive component of the polysaccharides. These bioactive components could be influenced by chemical components, molecular weight and method of extraction of the EPS. EPSWLD and EPSMLD also donated hydrogen ions which react with the DPPH radicals (Adebayo-Tayo et al., 2018). When DPPH and proton-donating substances are connected such as an antioxidant, the absorbance is reduced thereby scavenging the free radical. Antioxidant potential of EPS produced by *Weisella confusa* has been reported by Adebayo-Tayo et al. (2018). Zheng et al. (2006) reported that the total antioxidant activity of polysaccharide depends primarily on the configuration of the glycosidic bond and its structural characteristic. The sensitivity of free ferrous iron to oxygen which results in superoxide and ferric iron has been reported by Sahu and Gray (1997). Sahu and Gray (1997) also reported that the reaction of ferrous ion with superoxide hydrogen peroxide results in

Table 2. Sugar concentration and monosaccharide composition of the EPSWLD and EPSMLD.

Monosaccharide Name	EPSWLD			EPSMLD		
	Retention time	Area under Curve	Amount (mg/100g)	Retention time	Area under curve	Amount (mg/100g)
Ribose	8.656^{h}	1.62^{f}	2.06638^{f}	8.92^{h}	5.71^{c}	1.48119^{h}
Xylose	10.842^{g}	6.19^{b}	4.47902^{d}	10.65^{g}	4.43^{d}	1.82028^{g}
Arabinose	12.266^{f}	1.22^{g}	3.23573^{e}	12.27^{f}	4.46^{d}	2.25737^{f}
Rhamnose	13.660^{e}	1.06^{h}	3.55212^{e}	13.66^{e}	2.82^{e}	6.10709^{e}
Fructose	14.695^{d}	5.81^{c}	9.58819^{c}	14.69^{d}	8.10^{a}	7.73321^{d}
Glucose	16.094^{c}	1.93^{e}	39.8584^{a}	16.04^{c}	1.34^{f}	12.86967^{b}
Mannose	17.094^{b}	4.81^{d}	9.50729^{c}	17.09^{b}	7.16^{b}	8.70846^{c}
Galactose	18.056^{a}	6.50^{a}	20.44084^{b}	18.06^{a}	1.17^{g}	23.85038^{a}

Mean of values with the same superscript are not significantly different ($P \leq 0.05$).

Table 3. Antioxidant potential of the EPSWLD and EPSMLD.

Conc. (mg/L)	DPPH (%)		Total Antioxidant Capacity (%)		H_2O_2 (%)		FRAP (%)	
	EPSWLD	EPSMLD	EPSWLD	EPSMLD	EPSWLD	EPSMLD	EPSWLD	EPSMLD
0.5	38.5^{e}	37.5^{e}	1.21^{e}	0.41^{e}	50.6^{e}	33.1^{e}	1.23e	1.00^{e}
1.0	42.5^{d}	46.3^{d}	1.47^{d}	0.71^{d}	70.8^{d}	57.8^{d}	1.68d	1.04^{d}
2.5	59.4^{c}	53.3^{c}	1.64^{c}	0.82^{c}	80.7^{c}	64.2^{c}	1.75c	1.26^{c}
5.0	68.6^{b}	64.6^{b}	1.75^{b}	0.87^{b}	81.0^{b}	65.7^{b}	1.86b	1.72^{b}
10	73.4^{a}	65.6^{a}	1.80^{a}	1.42^{a}	88.5^{a}	78.6^{a}	1.89a	1.81^{a}
Ascorbic acid (10 mg/L)	27.9^{f}	30.8^{f}	0.87^{f}	0.25^{f}	37.9^{f}	21.8^{f}	0.59f	0.66^{f}

Mean of values with the same superscript on the same column are not significantly different ($P \leq 0.05$).

Table 4. Sensitivity test of the WLD and MLD to some antibiotics.

Antibiotics	Zone of Inhibition (mm)	
	WLD	MLD
Ceftazidine (30μg)	26	20
Cefuroxime(30μg)	0	0
Gentamicine (10μg)	0	0
Cefixime (5μg)	18	14
Ofloxacin (5μg)	22	20
Augmentin (30μg)	24	20
Nitrofurantoin (300μg)	14	8
Ciprofloxacine (5μg)	22	16
Amoxycillin (25μg)	18	14
Erythromycin (5μg)	28	22
Tetracycline (10μg)	26	24
Cloxacillin (5μg)	0	0
Cotrimoxazole (25μg)	24	20
Chloramphenicol (30μg)	26	24

Fenton reaction which leads into a hydroxyl radical by oxidizing biomolecules in the surroundings. The hydroxyl radical production is directly related to the concentration of copper or iron. The variation in antioxidant capacity of the EPSWLD and EPSMLD may be due to genetic modifications *L. delbrueckii* during mutagenesis (Saarela et al., 2011). This result shows that ferric ions had reducing ability for neutralizing free radicals to form a more stable product. Liu and Pan (2010) reported the reductive capability of *L. paracasei* and *L. plantarum* EPS.

The sensitivity of the WLD and MLD to some antibiotics is shown in Table 4. There was a significant difference ($P \leq 0.05$) in the antimicrobial sensitivity of the WLD and MLD to the antibiotics. 78.57% (11) of the WLD and MLD were susceptible while 18.75% (3) were resistant to the antibiotics. The susceptibility of WLD and MLD to the tested antibiotics ranged from 14f 0.00^{d}–28.0^{a}mm and 8f 0.00–24.0^{a} mm. The highest susceptibility was exhibited towards erythromycin, tetracycline and chloramphenicol. Generally, wild and mutant *Lactobacillus delbrueckii* were highly susceptible to chloramphenicol, cotrimoxazole, tetracycline, erythromycin and ceftazidine and were resistant to cefuroxime, gentamicin and cloxacillin.

The antibacterial activity of EPS produced by the LABs against some selected pathogenic microorganism is shown in Figure 3. There was a significant difference ($P \leq 0.05$) in the susceptibility of the test pathogen. The antibacterial activity ranged from 0.0 - 26.0 mm and 0.0–23.0 mm for EPSWLD and EPSMLD. *B. subtilis* and *S. aureus* had the highest susceptibility while *K. pneumonia* and *E. coli* were resistant to the EPS. *B. subtilis* was highly susceptible to all the EPS produced by the LAB strains.

The antimicrobial potential can be affiliated with the collection of organic acids, fatty acids, diacetyl and hydrogen peroxide (Dunne, 2001). Okereke et al. (2012) also reported that LAB isolated in their study prevents the growth of *S. aureus, E. coli* and *B. cereus*. Abdel-Bar et al. (1987) also reported that *L. bulgaricus* produces antimicrobial substance different from lactic acid and these substances are effective against to *S. aureus*. Lactobacilli are able to produce antimicrobial agents, such as

Table 6. Carcinoembryonic antigen (CEA) of the untreated and treated tumor induced mice.

Group	Treatments	CEA
1	CNT 1	0.01
	CNT 2	0.00
	CNT 3	0.00
	CNT 4	0.00
2	CNT+1	6.41
	CNT+2	6.29
	CNT+3	6.42
3	5F1	4.79
	5F2	4.97
	5F3	5.02
4	EPSWLD01	4.35
	EPSWLD02	4.06
	EPSWLD03	3.99
	EPSWLD04	4.11
5	EPSMLD01	4.23
	EPSMLD02	4.19
	EPSMLD03	4.17
	EPSMLD04	4.12

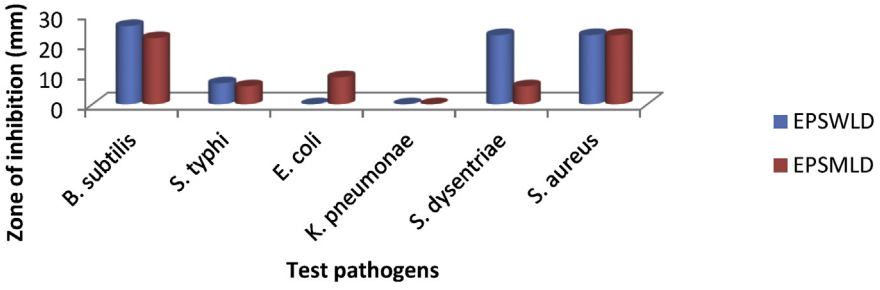

Figure 3. Antibacterial activity of EPSWLD and EPSMLD against some test pathogens.

Table 5. Antitumor and Immunomodulatory potential of EPSWLD and EPSMLD.

Groups	Cigarrete	EPSWLD	EPSMLD	% Flurouracil	Immunoglobulins (mg/dL)		
					IgG	IgA	IgM
Grp 1	-	-	-	-	68	67	64
Grp 2	+	-	-	-	105	67	63
Grp 3	+	-	-	+	108	84	70
Grp 4	+	+	-	-	122	95	89
Grp 5	+	-	+	-	126	98	97

Key: Florouracil is a standard drug for cancer treatment.

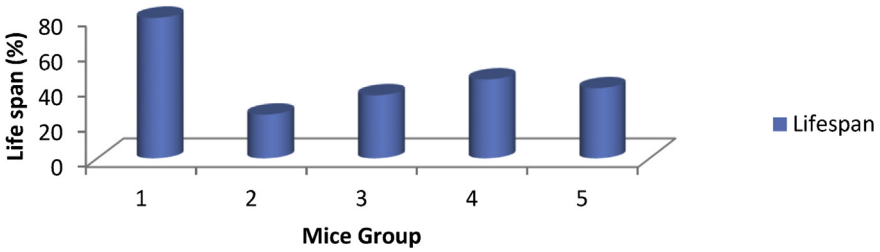

Figure 4. Life span of the untreated and treated tumor induced mice; Group 1 (non-tumor induced mice and untreated); Group 2 (tumor induced mice that is not treated); Group 3 (tumor induced mice treated with 5-fluorouracil acid); Group 4 (tumor induced mice treated with EPSWLD) and Group 5 (tumor induced mice treated with EPSMLD).

Table 7. Hematology Parameters of the untreated and treated mice.

Hematology parameters	Group 1 (c1)	Group 2 (c2)	Group 3 (c3)	Group 4	Group 5
RBCx10^{12}	4.7^{b}	3.0^{d}	3.7^{c}	5.6^{a}	3.8^{c}
WBC	37000^{c}	52000^{b}	65000^{a}	68000^{a}	51000^{b}
Platelet	21700^{b}	23400^{a}	15500^{c}	21300^{b}	11000^{d}
Lymph	70^{b}	42^{g}	53^{c}	77^{a}	70^{b}
Neut	70^{b}	58^{d}	61^{c}	72^{a}	64^{c}
PCV (%)	39.0^{b}	17.0^{d}	29.0^{c}	42.0^{a}	27^{c}
HB d/dl	12.9^{b}	5.66^{d}	9.68^{c}	13.1^{a}	10.67^{b}
MCH	2.74^{a}	1.89^{d}	2.61^{b}	2.34^{c}	2.80^{a}
MCHC	33.07^{b}	33.3^{b}	33.4^{b}	31.1^{c}	39.5^{a}
MCV	84.2^{a}	56.7^{d}	78.3^{b}	82.5^{a}	68.4^{c}

Mean of values with the same superscript are not significantly different ($P \leq 0.05$).

organic acids, hydrogen peroxide, diacetyl, short chain fatty acids and bacteriocins, against pathogens (Servin, 2004).

The immunomodulatory potential of tumor induced mice treated with EPSWLD and EPSMLD is shown in Table 5. There was a significant difference ($P \leq 0.05$) in immunoglobulins production by the treated mice. Group 5 (tumor induced mice treated with EPSMLD) had the highest IgG (126 ± 0.03 mg/ dL.), IgA level (98 ± 0.24 mg/dL) and IgM level (97 ± 0.05 mg/dL). This was followed in order by the IgG (122 mg/ dL), IgA level (95 mg/ dL) and IgM level (89 mg/ dL) of Group 4 (tumor induced mice treated with EPSWLD) and Group 3 (tumor induced mice treated with 5 –flurouracil acid). IgA level (67 mg/ dL) of Group 1(mice not administered with treatment) and Group 2(Tumor induced mice and not treated) are the same. The least IgM was recorded in group 2 (Tumor induced mice and not treated). There was no significant difference ($P \leq 0.05$) in the IgM level of Group 1 and Group 2.

Ability of EPSWLD and EPSMLD to stimulate IgG in the immune system of the mice may be due to the engulfment of macrophages by the EPS. There is a stimulation of serum glycoproteins to produce a sub-population of white blood cells called lymphocytes. Host immunity improvement based on Ig A production as a result of intake of LAB has been reported (Zhu et al., 2012; Qiu et al., 2012). Stimulation of immunoglobulin's in broiler chickens fed with LAB has been reported (Yang et al. (2012). Qiu et al., 2012) reported the immnomodulatory potential of one day old broilers fed with lactic acid bacteria strains and control diet has been reported.

The amount of carciniembryonic antigen was also investigated in the treated and untreated tumor induced mice (Table 6). Induced and un-treated mice displayed high level of CEA. Reduction in the CEA level of the induced and treated mice using 5-flurouracil acid as well as EPSWLD and EPSMLD was observed. The CEA level of the mice ranged from 0.00-0.01 ng/L in group 1. Group 2 had CEA which ranged from 6.29 – 6.42 ng/L. CEA level in Group 3 ranged from 4.79 - 5.02 ng/L. The CEA level of the mice in group 4 and 5 (3.99–4.35 ng/L and 4.12–4.23 ng/L) reduced compared to the CEA level of mice in group 2 and 3.

EPSMLD had the highest inhibitory potential on the tumor cell. This result is in agreement with the report of Yoo et al. (2004) on the CEA

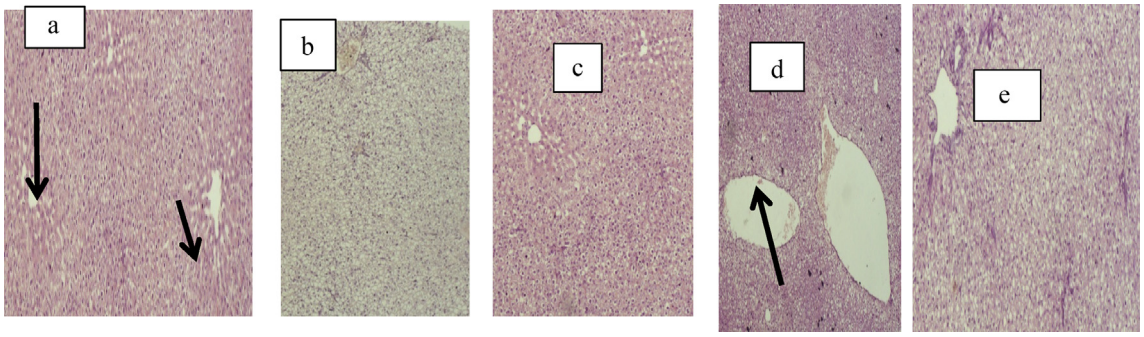

Figure 5. (a–e): Photomicrograph of the liver section of (a) non-tumor induced mice and untreated; (b) mice induced tumor and not treated; (c) tumor induced mice treated with 5-fluorouracil acid; (d) tumor induced mice treated with EPSWLD and (e) tumor induced mice treated with EPSMLD.

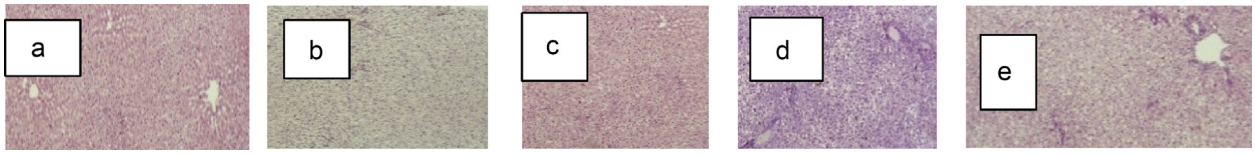

Figure 6. (a–e): Photomicrograph of the lung section of (a) non-tumor induced mice and untreated; (b) mice induced tumor and not treated; (c) tumor induced mice treated with 5-fluorouracil acid; (d) tumor induced mice treated with EPSWLD and (e) tumor induced mice treated with EPSMLD.

Inhibitory potential of EPS on tumor induced mice. The systolic effect in recovery of infection disease and tumor sing EPS has been reported by Zheng et al. (2006).

Ruiz-Brevo et al. (2001) reported that some bacterial EPS have been identified as biological Response Modifier (BMRs) with tumor rejection stimulatory activity. BMRs are agents that change the usual immune response and whose mode of action is induction of cytokines. Although, varieties of microbial EPS are immunomodulators, with activities for T-cells and macrophages while some polysaccharides are considered to be T-cell-independent antigens (Tzianabos, 2000). Doleyres and Laeroix (2005) reported the advantageous physiological effects of EPS on human health, such as antitumours activity, immunomodulating bioactivity and antimutagenicity. The ability of certain EPS from LAB on cytotoxic effect on cancer cells and their synergistically influence on the action of some chemotherapy drugs, such as camptothecin, to kill cancer cells has been reported (Cousin et al., 2012).

The lifespan of the tumor induced mice treated with EPSWLD and EPSMLD is shown in Figure 4. It was observed that Group 1 (non-tumor induced mice and untreated) had the highest lifespan (80 %) followed in order by Group 4 (tumor induced mice treated with EPSWLD) had a lifespan of 45 %. Group 5 (tumor induced mice treated with EPSMLD) had a lifespan of 40% while the least was observed in Group 3(tumor induced mice and treated with 5-flourouracil acid) had lifespan of 36% and Group 2 (tumor induced mice that is not treated) with life span of 25%. This may be due to the inhibition of DNA and RNA synthesis by the standard drug.

The hematological parameter of the mice induced with tumor is shown in Table 7. RBC, WBC, Lymphocyte, neutrophil, PCV and haemoglobulin of the untreated and treated mice ranged from 3.0–5.6 $\times 10^{12}$ %, 37000–68000 %, 42–77%, 58–72%, 17–42% and 5.66–13.1 d/dl respectively. Group 4 had the highest RBC, WBC, Lymphocyte, neutrophil, PCV and haemoglobulin level.

The Mean corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) of the experimental mice ranged from 1.89 - 2.80 and 31.1–39.5. The highest was recorded in group 1 and 4 respectively while the least was recorded in group 2 and group 5. The Mean corpuscular volume value ranged from 56.7 - 84.2 of which group 1 had the highest MCV value followed by group 4 while the least was found in group 2. The result showed that EPSWLD had significant increase in the numbers of RBC, PCV, Hb, Platelet and MCHC of mice exposed to tumor and treated with EPS.

PCV (Packed Cell Volume), RBC (Red Blood Cell), WBC (White Blood Cell), Hb (Haemoglobulin), PLT (Platelet), MCH (Mean corpuscular haemoglobin), MCHC (Mean Corpuscular haemoglobin Concentration) and MCV (Mean corpuscular volume).

The photomicrograph of the liver section of the untreated and treated mice with EPSWLD and EPSMLD is shown in Figure 5 (a–e).

The histology of liver in non-tumor induced mice and untreated is shown in Figure 5a. Pathological damage, abnormal lines and visible lesions were not observed. The histology of liver in mice induced with tumor and not treated is shown in Figure 5b. Peripotal inflammation was observed. Predominant abnormalities observed in the liver include vascular degeneration, necrosis of hepatocytes and severe interstitial Oedema. The histology of mice induced tumor and treated with 5-fluorouracil acid is shown in Figure 5c. There was a severe degeneration in the liver. The histology of mice induced tumor and treated with EPSWLD and EPSMLD is shown in Figure 5d and e. Moderate inflammation was observed. The visible and microscopic observation of the haematoxylin and eosin stained sections of liver of the trial animals revealed that the exposure of mice to cigarette caused pathological damage to their organs. These result into visible lesion and abnormality in the organs of the mice.

The photomicrograph of the lung section of tumor induced mice treated with EPSWLD and EPSMLD shown in Figure 6 (a–e). Figure 6a shows the histology of lung in non-tumor induced mice and untreated. There was a physiological damage in the lung. The photomicrograph of mice induced tumor and not treated is shown in Figure 6b. Abnormalities observed in the lung shows severe intestinal oedema and diffuse vascular degeneration. Figure 6c shows the micrograph of tumor induced mice treated with 5-fluorouracil acid. Diffused pulmonary oedema was observed. Histology of tumor induced mice treated with EPSWLD and EPSMLD is shown in Figure 6d and e. Moderate diffused pulmonary oedema was observed.

The serum glycoprotein stimulatory capability of the EPSWLD and EPSMLD on the mice which causes production of lymphocytes may be as a result of engulfment of macrophages by the exopolysaccharides. This stimulation lead to improvement in the heamatological parameter (BC, PCV, Hb, Platelet and MCHC) of the mice exposed to tumor and treated with EPSWLD and EPSMLD.

From the result Tumor induced mice shows the heamatopoietic function of the EPSWLD and EPSMLD. Effect of *Lactobacillus* and food on immune response has been evaluated Solis-Pereyra et al. (1997) and Gerritse et al. (1990). The values obtained in this were comparatively higher than that of Kabir et al. (2011) and Galip and Seyidoolu (2012). The result conformed to the observation of Ezema and Eze (2015) and Wallace et al. (2012) who reported that probiotics LAB producing EPS caused increase in total erythrocyte and leukocyte cell counts as well as marked increase in the percentage of lymphocytes and monocytes. Reticulocytosis occur as the initial response to stimulation of the erythropoietic system and is often characterized by macro cytic and hypochromic red blood cells with increased MCV and decreased MCH and MCHC values (Adamson and Longo, 2001). Therefore, EPSWLD and EPSMLD had stimulatory effect on immune system and it can be used to boost immune status.

4. Conclusion

FT-IR of the EPS produced by the wild type and mutant *Lactobacillus delbrueckii* shows varying degree of functional group which indicate the presence of EPS. The EPS had different sugar moiety and composition indicating heteropolysaccharide. The antioxidant activity of the EPSWLD and EPSMLD increases in a dose dependent manner. The EPS produced by wild type and mutant *Lactobacillus delbrueckii* had antibacterial and antitumor potential against the tumor induced mice. Tumor induced mice treated with EPS produced by EPSWLD and EPSMLD increased the RBC, platelet and WBC. The CEA level of the tumor induced mice treated with EPSWLD reduced compare to the tumor induced mice that is not treated.

Declarations

Author contribution statement

Bukola Adebayo-Tayo: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Racheal Fashogbon: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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