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Effects of *Lactobacillus* spp. isolated from the sap of palm tree *Elaeis guineensis* (palm wine) on cellular and innate immunity

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The isolation, characterization and effects of *lactobacillus* spp. from fresh palm wine was undertaken in this study. The aim of this study was to isolate and investigate the *in vivo* effects of the isolates on innate and cellular immune system. The result of the phenotypic and genotypic characterizations showed the presence of *Lactobacillus brevis, Lactobacillus paracasei subsp. Tolerans, Lactobacillus paracasei* and *Lactobacillus yonginensis,* which are members of *Lactobacillus* spp., isolated. The effects of the isolates on innate and cellular immunity were investigated, using two models namely: *in vivo* leukocytes mobilization rate (LMR) and delayed type hypersensitivity response (DTHR). The different isolates had very significant effect on cellular immunity as represented by their ability to stimulate delayed type hypersensitivity response in treated rats. They caused a percentage increase of 300, 600, 600, 500 and 650% for *L. brevis, L. paracasei subsp. Tolerans, L. paracasei, L. yonginensis* and positive control respectively when compared with the negative control. Similarly, the various isolates also affected significantly the innate immune system through their marked influence on the total leukocyte count according to the result of the experiment. They were able to produce a percentage increase of 213, 204, 188.6, and 152.5% for *L. paracasei, L. yonginensis, L. paracasei subsp. Tolerans* and *L. brevis* respectively; while the positive control produced 83.8% increase when compared with the negative control. The result of this study showed that the four *lactobacillus* spp. isolated from fresh palm wine significantly affected the innate and cellular component of the immune system positively.

Key words: Delayed type, hypersensitivity response, innate immunity, cellular immunity, leukocyte mobilization.

INTRODUCTION

The popular quotation by Hippocrates adjudged the father of medicine “let food be thy medicine, and let medicine be thy food” hundreds of years ago is now gaining popularity. Thus, in recent time the idea of food having medicinal value has been given the name “functional food”. The recent trend of an increasing research in the area of probiotics with demonstrated therapeutic evidence has also given impetus to the

concept of functional food. There is now an increased demand for food/products that have the capacity to enhance health, beyond providing basic nutrition, since humans are now aware of the relationship between diet, lifestyle and good health. This functionality of food has been linked to the presence of certain beneficial bacterial especially lactic acid bacteria (LAB) that are generally recognized as safe (GRAS) bacteria. Besides the nutritional values, ingestion of Lactic acid bacteria (LAB) and their fermented foods has been suggested to confer a range of health benefits including immune system modulation, increased resistance to malignancy, and infectious illness (Soccol et al., 2010). These bacteria have also been linked with improvement in lactose utilization, anti-cholesterol, and production of bacteriocin an effective antimicrobial compound that is of immense benefit to man (Krishnendra et al., 2013; Aween et al., 2012). Some of the benefits of these bacteria have been exploited in its co-administration with antibiotics. They are shown to improve antibiotic therapy as they reduce microbial adhesion and growth by bacteriocins or other inhibitory compounds, possess immunomodulatory properties, and improve intestinal barrier integrity (Reid, 2006). It also promote the recovery of commensal microbiota and increase treatment tolerability in patients on antibiotic therapy (Boyanova and Mitov, 2012). It has become obvious that the need for alternative/ advancement in antibiotic therapy cannot be over emphasized. This is because antibiotics are now losing their effectiveness, particularly due to overuse, misuse and subsequently to the increasing development of antibiotic resistance (D`Souza et al., 2002). These beneficial bacteria have been seen recently as valuable adjunct to antibiotic therapy since continued or excessive use of antibiotics is known to disrupt the normal micro flora of the human body, which they have the capacity to reverse. They are also a veritable tool in the handling of some of the side effects of antibiotics therapy such as antibiotic-associated diarrhoea (AAD) (Cremonini et al., 2002; Armuzzi et al., 2001; Vanderhoof et al., 1999; Arvola et al., 1999).

Palm wine is the collective name for a group of alcoholic beverages, whitish in color and obtained through natural fermentation of the sap of *Elaeis guineensis* (Uzochukwu et al., 1991). Yeast, lactic acid bacteria and acetic acid bacteria are involved in the production of palm wine. The beverage is linked with high content of amino acid, potassium, zinc and iron (Carousel, 2015). It also contains B1, B2, B3, and B6 vitamins and have been linked with increased sperm and breast milk (Mbuagbaw and Noorduyn, 2012). Palm wine is a drink that is common in Southern part of Nigeria because of its central role in most traditional ceremonies. Its use in this ceremonies/social functions is due to its alcohol intoxicating effect. Palm wine also have been linked with somewhat health benefits where amongst some locals, children with suspected cases of measles infection are given fresh palm wine and it's believed to reduce reasonably high fever associated with measles within few hours. It is used extensively as a galactogogue (substances that are capable of stimulating breast milk production) in women newly delivered of their babies. These two common uses of the beverage in our local communities motivated us to seek possible scientific explanation to ascertain the veracity of the claim and to possibly look at the best way to exploit the benefit(s).

MATERIALS AND METHODS

The equipment and instruments used include hot air oven (Genlab thermal engineering, Transhouse lane Widness, Cheshire), autoclave (Shenan LDZX-50FB, England), incubator (Genlab thermal engineering, Transhouse lane Widness, Cheshire), binocular microscope (Olympus), and anaerobic jar (Oxoid, UK). Also included were refrigerator, weighing balance (Adventurer, Ohaus Corp. Pine brook, USA), wire loop, sample containers, micropipette, syringe and needle, surgical gloves, measuring cylinders, Petri dishes, beakers, conical flask, Durham tube, distilled water, Deionized water, 70% ethanol, gentian violet, lugol's iodine, safranin, malachyte green, hydrogen peroxide, glucose, sucrose,lactose, and Linex capsule (lyophilized lactobacillus capsules). Ovalbumin, goat anti-mouse IgG^{Fab} HRP(Southern biotech, USA), Goat anti-mouse $IgG1^{Fab}$ HRP(Southern biotech, USA), Goat anti-mouse $IgG2a^{Fab}$ HRP(Southern biotech, USA), Fat free milk, Tween-20, Phenol red, Immersion oil, Dmso solution, dibasic sodium phosphate, citric acid solution, and TMB substrate tablet (Sigma-Aldrich USA) were also part of the materials used. Sodium hydroxide and Tetraoxosulphate vi acid,animals and organisms used in the study include Sap of the oil palm tree (*E. guinneesis)* palm wine, Wistar albino mice (28-30 g), Wistar albino rat (90-120 g), lactic acid bacteria, modified De Man Rogosa and Sharpe (MRS) Agar (Himedia, India) were also used.

Collection of palm wine

The fresh palm wine samples were collected in the morning around 6:30 am from a local palm wine tapper, Mr Anthony Idoko, in Onicha-Enugu Ezike in Igbo Eze North local Government Area of Enugu state. The samples were kept under cold conditions, using ice pack to reduce the rate of fermentation while being transported to the laboratory.

Preparation of media and culturing of lactic acid bacteria

The MRS agar media used were prepared following manufacturer`s specifications. The test organisms were cultivated from *E. guineensis* sap (Palm wine) using streak plate method. A wire loop was used to collect a loopful of the homogenized Palm wine samples and streaked on the surface of solidified MRS media under aseptic conditions. The inoculated media were incubated

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anaerobically using anaerobic jar at 37°C for 24 to 48 h.

Isolation of lactic acid bacteria

A loopful of distinct colonies formed on the solidified media during culturing were subculture on sterile modified MRS agar plate by quadrant streaking method, under aseptic conditions. After streaking, all the petri dishes were incubated at 37°C for 24 to 48 h. After the incubation, colonies were sub cultured on MRS agar to obtain pure cultures. The purified isolates were streaked on MRS agar slants and stored at 4°C for further use.

Phenotypic characterization of culture

The isolates were presumptively identified based on cultural, morphological and some biochemical characteristics. The parameters investigated included colony morphology, Gram reactions, endospore formation, catalase production, motility, and sugar fermentation. The results were compared to Holt et al. (1994) Bergey's Manual of Determinative Bacteriology.

Genotypic characterization of the Isolate

DNA extraction and PCR amplification of the isolates

DNA Extraction was carried out on test organisms' isolated from Palm wine using the Jena Bioscience Bacteria DNA Preparation Kit (http://www.jenabioscience.com).

Polymerase chain reaction was carried out to identify the suspected lactic acid bacteria isolated from palm wine using the primer pair BSF8 (AGAGTTTGATCCTGGCTCAG) and BSR534 (ATTACCGCGGCTGCTGC) The primer pair are lactic acid specific. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25 pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5 µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture.

Thermal cycling was conducted in a Peltier thermal cycler (PTC100) (MJ Research Series) for an initial denaturation of 95°C for 15 min, followed by 35 amplification cycles of 30 s at 95°C; 1 min. at 58°C and 1 min 30 s at 72°C. This was followed by a final extension step of 10 min at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 h 30 min. After electrophoresis, DNA bands were visualized using ethidium bromide staining. 100bp DNA ladder was used as DNA molecular weight standard.

Identification of the isolates

All the isolates were identified using 16S rRNA. All PCR products were purified and sent to Epoch Life science (USA) for Sanger sequencing. The corresponding sequences were identified using the online blast search at http://blast.ncbi.nlm.nih.gov/Blast.cgi

Immunological studies

Grouping and dosing of the animals

The animals were grouped into 13 groups of 5 animals in a group. The first 11 groups received 3×10^7 CFU and 9×10^7CFU of each of the isolates determined by 0.5 McFarland standard for experiments involving mice and rats respectively. The 12th and the 13th group represent positive and negative control groups respectively. The positive control receives linex capsule a brand of lyophilized *Lactobacillus* spp. at a dose of 7.2×10^5 and 1.2×10^5 for experiments involving rats and mice respectively.

Studies on delayed type hypersensitivity response (DTHR)

Delayed type hypersensitivity was induced in rat using sheep red blood cells (SRBC) as antigen. Animals were sensitized by subcutaneous injection of 0.02 ml of 1×10^9 cells ml^{-1} SRBC (day 0) in the plantar region of right hind foot paw and challenged on day 5 by subcutaneous injection of same amount of antigen into the left hind paw. The oedema produced by antigenic challenge in the left hind paw was taken as the difference in the paw thickness before and 24 h after the challenge. The paw thickness was measured by utilizing volume displacement of water. LAB isolates were administered 3 days prior to sensitization and continued at a daily dose of 9×10^7 cfu until the challenge (Naved et al., 2005; Shinde et al., 1999).

Study on in vivo leucocyte migration rate

The method of Ribeiro et al., (1991) was utilized in the *in vivo* leucocyte migration study. The *in vivo* leucocyte migration was induced by inflammatory stimulus. One hour after oral administration of the 3×10^7 cfu of the LAB isolates, each mice in the groups received intraperitoneal injections of 0.5 ml of 3% (w/v) agar suspension in normal saline. Four hours later, the mice were sacrificed and the peritoneum washed with 5 ml of a 5% solution of EDTA in phosphate buffered saline (PBS). The peritoneal fluid was recovered and total (TLC) performed on the perfusates. The cells were counted with the help of Atacus 30 haematology autoanalyser

Statistical analysis

The statistical analysis was done using Graph Pad prism version 5.0. One-way ANOVA followed by Post-hoc Dunnet was used to compare mean ± SEM, and values were considered significant at $p < 0.05$.

RESULTS

Morphological and biochemical characteristics of isolated lactic acid bacteria from palm wine

The morphological, cultural and biochemical characteristics of the isolated bacteria from palm wine are as shown in the (Table 1).

Genotypic characterization and blasted sequence results of the isolates

The result of the blasted sequenced results showed the presence of four species of Lactobacillus namely: *L. brevis*, *L. paracasei subsp. Tolerans, L. paracasei* and *L. yonginensi*.

Table 1. Morphological and biochemical characteristics of the isolates.

Suspected organism	Gram stain	Endospore test	Catalase	Motility	Glucose fermentation
1	+	-	-	-	+/G $^{-ve}$
2	+	-	-	-	+/G $^{-ve}$
3	+	-	-	-	+/G $^{-ve}$
4	+	-	-	-	+/G $^{-ve}$

(+), positive; (-), negative; +/G $^{+ve}$, gas production= heterofermentation; +/G $^{-ve}$, without gas production= homofermentation.

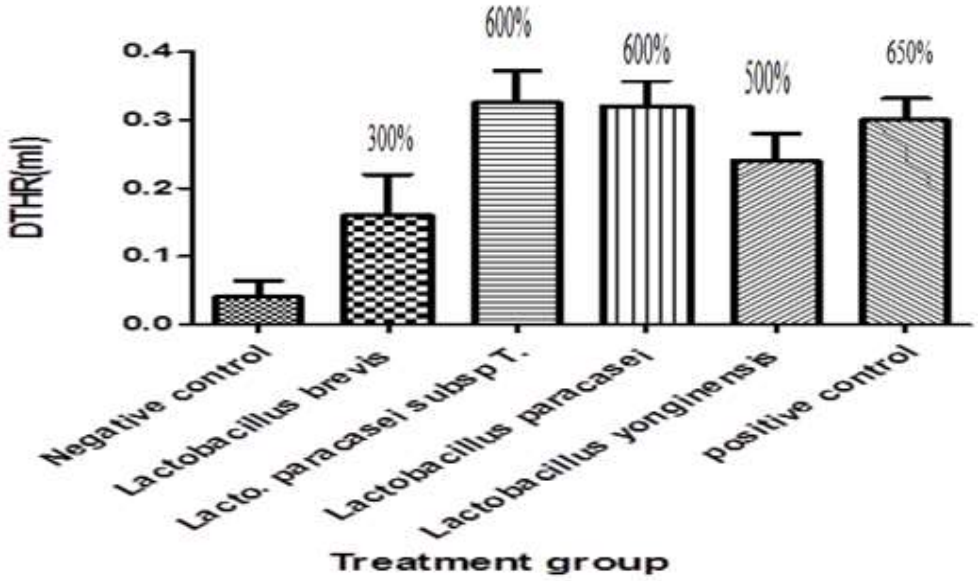

Figure 1. The effect of Lactobacillus spp. on delayed type hypersensitivity response (DTHR) in rats.

The effects of *Lactobacillus* spp. on Delayed type hypersensitivity response in rats (DTHR)

Figure 1 shows the effect of *Lactobacillus* spp. on DTHR in rats. The four *Lactobacillus* spp. produced a significant percentage increase in stimulation of DTHR in rats challenged with sheep red blood cell as antigen when compared with the negative control group. It produced a percentage increase of between 600 to 300% when compared with the negative control.

The effects of *Lactobacillus* spp. on Total Leukocytes count (TLC) on albino mice

The effect of *Lactobacillus* spp. on total leukocyte count (TLC) that mobilized to the site of injury was determined from the peritoneal washout of the sacrificed animals and the TLC determined with the aid of Atacus 30 haematology autoanalyser. The result showed that there was significant percent increase on the amount of Leukocytes mobilized to the site of injury for the *Lactobacillus* spp. isolates (Figure 2).

DISCUSSION

Alcohol consumption has always been a public health problem. Globally, alcohol use is the third leading risk factor for poor health and causes an estimated 2.5 million deaths per year (Mikolajczyk et al., 2016). However, most of the reports on deleterious effect of alcohol on our health are linked to synthetic alcohols and not necessarily from natural alcoholic beverages. This is because in the case of palm wine, when freshly tapped, palm wine has little to no alcoholic content and is said to offer several nutritional benefits to the human body. The main ingredient of the fresh palm sap is sucrose, which is about 12 to 15% by weight with little reducing sugar such as glucose, fructose, maltose and raffinose, in addition to

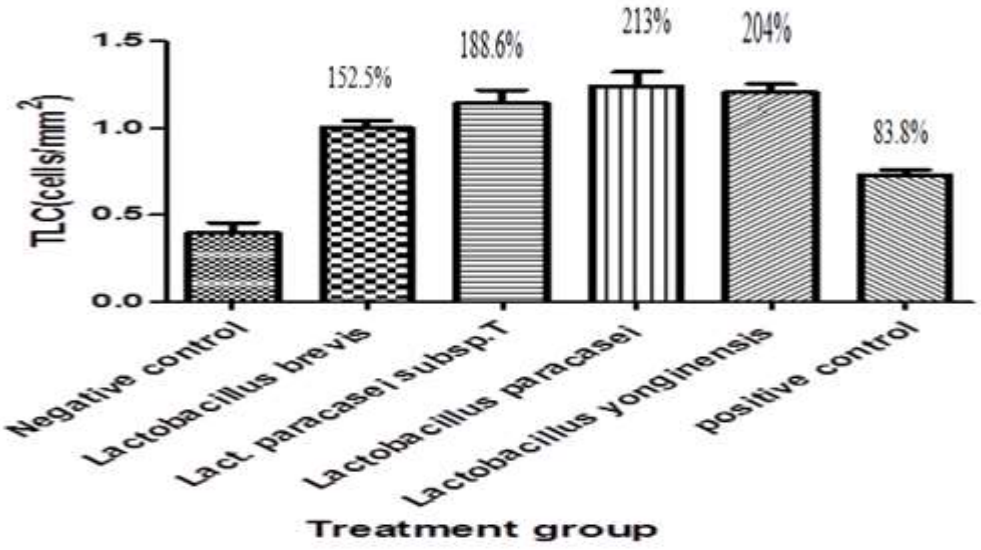

Figure 2. The effects of *Lactobacillus* spp. on Total Leukocytes count (TLC) on albino mice.

sugar, the sap also contains protein, fat and mineral matter (Ezeagu et al., 2003). The sap can be regarded as a safe (GRAS) and beneficial lactic acid bacteria spp.

This study isolated, characterized and investigated the effects of lactobacillus spp. from palm wine on the cellular and innate immune systems. *Lactobacillus brevis, L. paracasei subsp. Tolerans, L. paracasei* and *L. yonginensis* were the species of the genera *Lactobacillus* isolated from the sampled Palm wine. Lactobacilli are of significant technological importance as they are involved in the manufacturing of several fermented and non-fermented foods, and have been used as probiotics due to their health-promoting effects. Probiotic lactobacilli have been associated with the prevention and treatment of gastrointestinal disorders, such as rotavirus diarrhea, antibiotic-associated diarrhea, and travelers' diarrhea, and have been suggested as potential therapeutic agents against irritable bowel syndrome and inflammatory bowel disease (Lomax and Calder, 2009). *Lactobacillus* species are the most extensively studied Lactic acid bacteria that have gained importance in the clinics as a therapeutic agent where lyophilized form of the organism is used in the treatment of diarrhea. The ability of lactobacilli to adhere to the gastrointestinal mucosa has been suggested to influence their interaction with the host and the other bacteria present, by affecting the local microbial composition and/or by stimulating the host's immune system (Qin et al., 2009). The effects of the isolates on cellular and innate immune systems were investigated utilizing models that represent the two different components of immunity namely innate immunity (*In vivo* leukocyte mobilization rate); cell mediated immunity (delayed type hypersensitivity response). The isolates *L. brevis, L. paracasei subsp. Tolerans, L. paracasei* and *L. yonginensis* produced a percentage stimulation of DTHR by 300, 600, 600 and 500 respectively when compared with the negative control (Figure 1). Delayed hypersensitivity reactions are inflam-matory reactions initiated by mononuclear leukocytes. The term delayed is used to differentiate a secondary cellular response, which appears 48 to 72 h after antigen exposure, from an immediate hypersensitivity response, which generally appears within 12 min of an antigen challenge (Abramson et al., 2018). These reactions are mediated by T cells and monocytes/ macrophages rather than by antibodies. They are also termed type IV hypersensitivity reactions. Delayed hypersensitivity is a major mechanism of defense against various intracellular pathogens, including mycobacteria, fungi, and certain parasites, and it occurs in transplant rejection and tumor immunity (Abramson and Kaliner, 2018). The cellular events that result in delayed hypersensitivity reactions primarily involve T cells and macrophages. First, local immune and inflammatory responses at the site of foreign antigen up-regulate endothelial cell adhesion molecule expression; promoting the accumulation of leukocytes at the tissue site. The antigen is engulfed by macrophages and monocytes and is processed and presented to a T cell that has a specific receptor for that processed antigen (Abramson and Kaliner, 2018).

The various species of lactobacillus isolated from the study showed a significant increase of 152.5, 188.6, 213 and 204% for *L. brevis, L. paracasei subsp. Tolerans, L. paracasei* and *L. yonginensis* respectively for leukocyte that migrated to the site of injury (Figure 2). Circulating blood leukocytes are required to migrate to sites of tissue injury and infection with the principal aim of eliminating the primary inflammatory trigger and contributing to tissue repair. In innate immunity, this process is largely initiated by pathogen-associated molecular patterns (PAMPs),

released by invading microorganisms, and damage-associated molecular patterns (DAMPs), derived from damaged and/or dead-cells, or in response to tissue and/or cellular stress (Medzhitov, 2008). In addition, antigens, largely through activation of resident memory T cells, can trigger recruitment of leukocytes via secretion of various primary inflammatory cytokines. Tissue sentinel cells, including mast cells, macrophages, and dendritic cells (DCs), play a key role in detection of such danger signals and can release a wide range of pro-inflammatory mediators to promote leukocyte recruitment. The high values recorded from the result is in agreement with the postulation that the success of lactobacillus spp. as an immunomodulator is linked to its ability to bind to PRR expressed on immune cells and the cascade of other events that follows (Wells et al*.,* 2010; Abreu, 2010; Kawai and Akira, 2009).

The use of fresh palm wine by local dwellers in the communities to treat children with measles has been an age long practice. Here, children with suspected cases of measles are usually given some amount of fresh palm wine orally and most times the high fever associated with measles subsides immediately. The result of the effect of the isolates on the respective aspects of the immune system may now give us an insight and possible scientific explanation on the use of fresh palm wine in children with measles. The result clearly showed that the isolates had a marked effect on cellular immunity (T-cells) and innate immunity. It is established that measles is an infection involving intra-cellular pathogens (viruses), and intracellular pathogens are absolutely handled by T-lymphocytes. Cell-mediated immunity attributable to T cells is the principal mechanism whereby intracellular organisms are eliminated by macrophages activated by γ-interferon derived from T cells. This beverage use for measles is strongly believed that it is linked to its ability to harbor these beneficial immune stimulatory organisms.

It is also an old practice by locals to administer fresh palm wine to women newly delivered of their babies as lactation enhance. Prolactin the hormone that controls lactation was originally identified as a neuroendocrine hormone of pituitary origin. However, its synthesis is not limited to the hypothesis since numerous extra pituitary tissues also express this protein, including the placenta, ovary, testis, mammary gland, skin, adipose tissue, endothelial cells, and immune cells (macrophages, natural killer cells, T-cells and B- cells) (Harvey et al*.,* 2012). Therefore, it is obvious that the significant stimulation of both the T cell and the innate immune system by these lactic acid bacteria found in this natural beverage may be the scientific explanation of its lactation enhancing potential. All these enumerated health benefits of *Lactobacilus* spp. and many other industrial benefits can be maximally harnessed without the obvious public health challenges of alcohol. This is achievable through moderate intake of sweet non-fermented/slightly fermented *E. guinneesis* sap (Palm wine) since alcoholic fermentation is a process that is time dependent, and the process convert sugar rich beverage by these beneficial bacteria to alcohol and finally to ethanoic acid.

Conclusion

Fresh *E. guinneesis* sap (palm wine) harbor *Lactobacillus* spp. with Immune enhancing property and these enormous benefits can be exploited through moderate intake of the beverage in a fresh and non-fermented state.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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