

*Full Length Research Paper*

## **Study of the antibody stimulation potentials of *Lactobacillus* spp isolated from palm wine**

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**In this study lactic acid bacteria were investigated for their presence in fresh palm wine and their effects on humoral immunity examined. The bacteria were isolated from fresh palm wine in a tenfold serial dilution. The isolates were purified by repeated subculture on De Man Rogosa Sharpe (MRS) agar and characterized phenotypically and genotypically. The identified *Lactobacillus* spp. were investigated for their effects on antibody (humoral immunity) - IgG, IgG1 and IgG2a secretion using sheep red blood cell as antigen (SRBC). *Lactobacillus* spp. were identified as *Lactobacillus brevis*, *Lactobacillus paracasei* subsp. *tolerans*, *Lactobacillus paracasei* and *Lactobacillus yonginensis*. The isolates produced no significant effect on IgG antibody after 4 days' post-secondary challenge. However, they had significant percentage stimulation at 9 days post-secondary challenge of 100.2, 122.9, 106.4 and 118.3% for *L. brevis*, *L. paracasei* sub. *tolerans*, *L. paracasei*, and *L. yonginensis*, respectively. The isolates had marginal and somewhat suppressive effects on IgG1 and IgG2a at both 4 and 9 days' post-secondary challenge. The results show that fresh palm wine contains *Lactobacillus* spp. capable of stimulating antibodies production.**

**Key words:** Humoral immunity, antibody, palm wine, *Lactobacillus* spp.

### **INTRODUCTION**

*Lactobacillus* spp. are important class of bacteria that have been extensively studied by scientists in recent times. They are generally regarded as safe (Gras) bacteria hence exploited in several ways to the benefit of man. *Lactobacillus* have been linked with the production

of antimicrobial substances, antibiotic resistance patterns, improving digestive ability and antibody mediated response with demonstrable efficacy and safety (Hou et al., 2015; Wang et al., 2012). Studies over the years have proved that *Lactobacillus* spp are potent

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modulators of the immune system.

They are able to modulate both the adaptive and innate components of the immune system through the regulation of the functions of dendritic cells, macrophages, T and B lymphocytes. The success of this class of bacteria as an immunomodulator is linked to its ability to bind pattern recognition receptors (PRR) expressed on immune cells. These PRR in turn recognize conserved molecular structure known as microbe associated molecular patterns (MAMPS) and signal to induce the production of cytokines, chemokines and other innate effectors (Wells et al., 2010; Abreu, 2010; Kawai and Akira, 2010). Recent studies have shown that probiotics (e.g lactic acid bacteria) are becoming popular as an option in the treatment of inflammatory disorders (Evrard et al., 2011; Kwon et al., 2010). They also influence positively though indirectly, the regulatory T cells (Treg) by providing a favourable environment (Petersen et al., 2012). *Lactobacillus* spp administration/studies *in vivo* and *ex vivo* produced significant increase in all functions of peritoneal macrophages viz, increased microbicidal and phagocytic activities and increased production of cytokines induced by macrophages (Marranzino et al., 2012). *Lactobacillus lactis* strains commonly found in food products directly stimulate plasmacytoid dendritic cells (pDC) resident in the intestinal draining mesenteric lymph nodes to produce not only type 1 IFN but also IFN- $\lambda$  and augment the capacity of pDC to induce CD4+CD25+Treg generation (Jounai et al., 2012). *Lactobacillus acidophilus* increases the cytotoxic activity of natural killer cells (Corthesy et al., 2007). These beneficial and safe bacteria stimulate innate immune system in immune deficient individuals (Delcenserie et al., 2008) and also in immune deficient elderly persons where they stimulate both phagocytic activity of macrophages and natural killer cell function (El-Gaaly et al., 2016).

It is an established fact that *Lactobacillus* spp belong to a group of bacteria that have been extensively studied because of their enormous health benefits. The lyophilized forms of these bacteria are now used as therapeutics in the management of diarrhea. This study derived its uniqueness from the fact that these bacteria was isolated from a natural alcoholic beverage. *Elaeis guineensis* (Palm tree) sap (Palm wine) was studied for the presence of these important bacteria and their effect on humoral immunity. Palm wine is a whitish liquid produced as a result of natural fermentation by acetic acid bacteria, lactic acid bacteria and yeast (Santiago-Urbina and Ruiz-Teran, 2014). These organisms usually cause the breakdown of sugar in the sap into alcohol and other products (Obire, 2005). These organisms are responsible for the sour taste experienced on this beverage over time as the tapping process progresses. The chemistry of what happens is that at the point of tapping the beverage is rich in sugar hence very sweet. The activities of these beneficial bacteria tend to convert

the sweet beverage into alcohol and ethanoic acid (sour taste experienced after about 24 h of fermentation) in a time dependent process (Eze et al., 2019). In West African sub-regions, this sweet and fresh alcoholic beverage is linked with some health benefits such as increased sperm in men and breast milk production in women, and high content of B1, B2, B3, and B6 vitamins (Mbuagbaw and Noorduyn, 2012). The beverages also possess high content of amino acid, potassium, zinc and iron (Carousel, 2015).

In this study, we investigated the presence of *Lactobacillus* as they are suspected to be involved in the fermentation of palm wine and the effect of the isolates on humoral immunity was determined.

## MATERIALS AND METHODS

### Sources of *Lactobacillus*

Fresh palm wine from oil palm tree *Elaeis guineensis*, Linex capsule (lyophilized lactobacillus capsule) Sandoz Pharmaceutical Slovenia.

### Sources of antibodies and antigens used

Ovalbumin (Sigma-Aldrich, USA), Goat anti-mouse IgGFab HRP (Southern biotech, USA), Goat anti-mouse IgG1Fab HRP (Southern biotech, USA), Goat anti-mouse IgG2aFab HRP (Southern biotech, USA), albino mice (28-30 g), Sheep red blood cell (SRBC).

### Collection of palm wine

The fresh palm wine samples were collected at about 6:30 am by Mr Anthony Idoko a local Palm wine tapper from Onicha Enugu Ezike, Igbo- Eze North Local government area of Enugu State, Nigeria. 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 isolation of *Lactobacillus* spp

The media were prepared following the manufacturer's specifications. The test organisms were isolated from Palm wine using quadrant streak plate method. A wire loop was used to collect a loopfull of the homogenized ten- fold serial dilution of the fresh Palm wine samples and streaked on the surface of sterile modified MRS media under aseptic conditions. The inoculated media were incubated using anaerobiosis generator at 37°C for 24-48 h. After the incubation, distinct colonies were subcultured 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 the 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 Isolates

#### DNA extraction and polymerase chain reaction (PCR) amplification

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<sub>2</sub>, 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. 100 bp 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 6 groups of 5 animals (mice) per group. The first 4 groups received 0.2 ml of 3×10<sup>7</sup> cfu/ml of each of the isolates determined by 0.5 McFarland standard. The 5th and the 6th group represent positive and negative control groups respectively. The positive control received linex capsule (0.2 ml) a brand of lyophilized *Lactobacillus* spp. at a dose of 1.2 × 10<sup>5</sup> cfu/ml.

##### Studies on humoral antibody HA response

This is a slight modification of the method by Sharma et al. (1996). Mice were immunized by an intraperitoneal (ip) injection of 0.2 ml of 1×10<sup>9</sup> SRBC ml<sup>-1</sup> on (day 0) and challenged by similar i.p injection of the same amount on day 5. The animals were bled and the serum analysed for the antibody titre by ELISA on days 10 and 15. Briefly 1 mg/ml of ova in bicarbonate buffer (pH 9.6) was prepared and used to coat the 96 well ELIZA plates and the plates incubated at 4°C for about 14 h. The plates were washed with PBS-Tween (0.05% Tween-20 in 0.1 M PBS). The plates were blocked with 20 µl/well of 5% v/v solution of fat free milk in PBS-Tween and

incubated at room temperature for 1 h. The plates were washed 3 times with PBS-Tween. The sera from the animals were diluted 1:20 with 2% fat free milk and added 100 µl/well in duplicates. The plates were washed with PBS-Tween. 100 µl of 1:1000 dilution of HRP-antigoat Immunoglobulin were added to the wells and incubated for 1 h at room temperature. The plates were washed four times with PBS-Tween. 100 µl of the quenching agent TMT were added per well and allowed for at least 15-20 min and the plates read in ELIZA machine at 405 nm. The LAB isolates were administered 3 days prior to immunization and continued on a daily dose of 3×10<sup>7</sup> cfu/ml until the 14th day. The immunoglobulin studied included IgG, IgG1 and IgG2a.

#### Statistical analysis

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

## RESULTS

### The morphological and biochemical characteristics of the isolated *Lactobacillus*

The morphological and biochemical characteristics of the isolated *Lactobacillus* are as shown in Table 1.

### Genotypic and blasted sequence results of the isolates

The result of the blasted sequenced showed the presence of four species of *Lactobacillus* namely: *L. brevis*, *L. paracasei* subsp. *Tolerans*, *L. paracasei* and *L. yonginensi*. Primer pairs - BSF8 (AGAGTTTGATCCTGGCTCAG) and BSR534 (ATTACCGCGGCTGCTGC) was used to identify the suspected lactic acid bacteria isolated from the palm wine.

### The effect of *Lactobacillus* spp. on antibody secretion

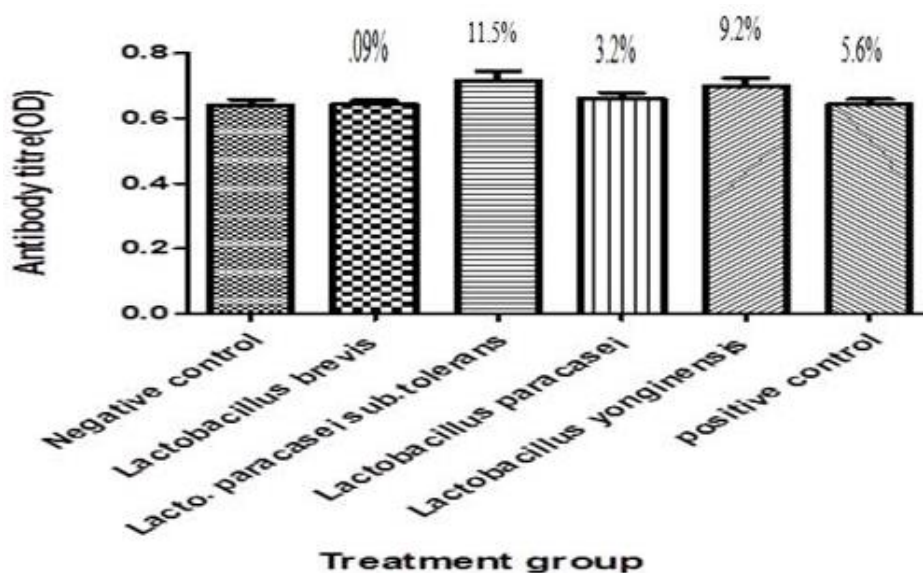
Figures 1 and 2 shows the effect of *Lactobacillus* spp on IgG antibody secretion 4 days and 9 days post-secondary challenge in mice. The isolates produced insignificant effects on IgG antibody secretion 4 days post-secondary challenge; however, there was a significant increase of 100.2, 122.9, 106.4 and 118.3% for *L. brevis*, *L. paracasei* subsp. *Tolerans*, *L. paracasei* and *L. yonginensi* respectively when compared with the negative control. The effects of *Lactobacillus* spp on IgG1 and IgG2a secretion 4 and 9 days post-secondary challenge is as shown in Figures 3 to 6.

*L. paracasei* subsp. *Tolerans*, *L. paracasei* and *L. yonginensis* produced a significant percentage increase of 30.1, 48.6 and 31.7% on the albino mice 4 days post-secondary challenge while only *L. yonginensis* produced a significant percentage increase of 39% 9 days

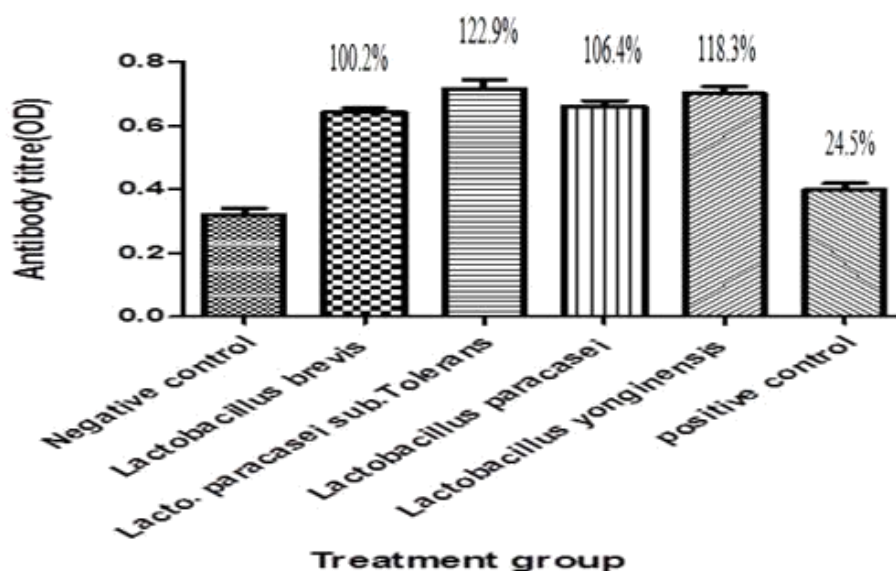
**Table 1.** Morphological and biochemical characteristics of the isolates.

Suspected Organism	Gram stain	Endospore Test	Catalase	Motility	Glucose Fermentation
I	+	-	-	-	+/-ve
II	+	-	-	-	+/-ve
III	+	-	-	-	+/-ve
IV	+	-	-	-	+/-ve

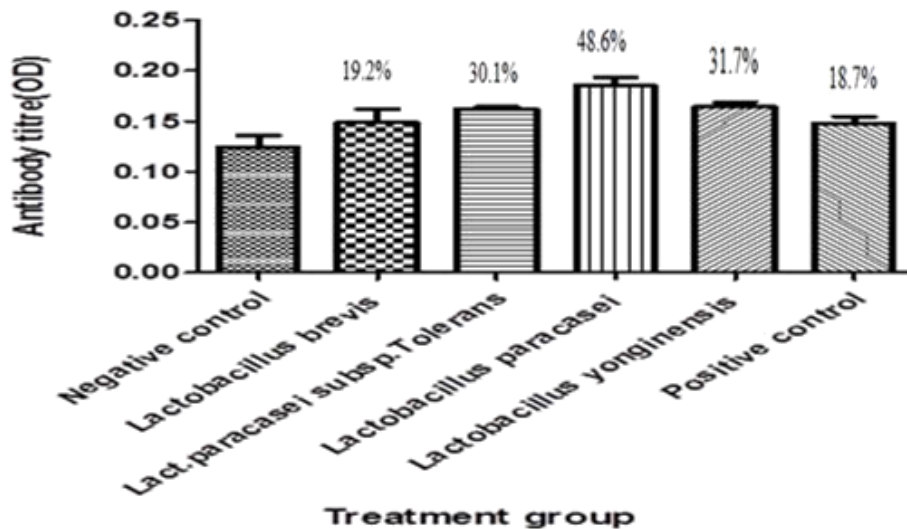
Positive; (-) negative; +/-ve gas production = heterofermentation; +/-ve, without gas production = homofermentation.



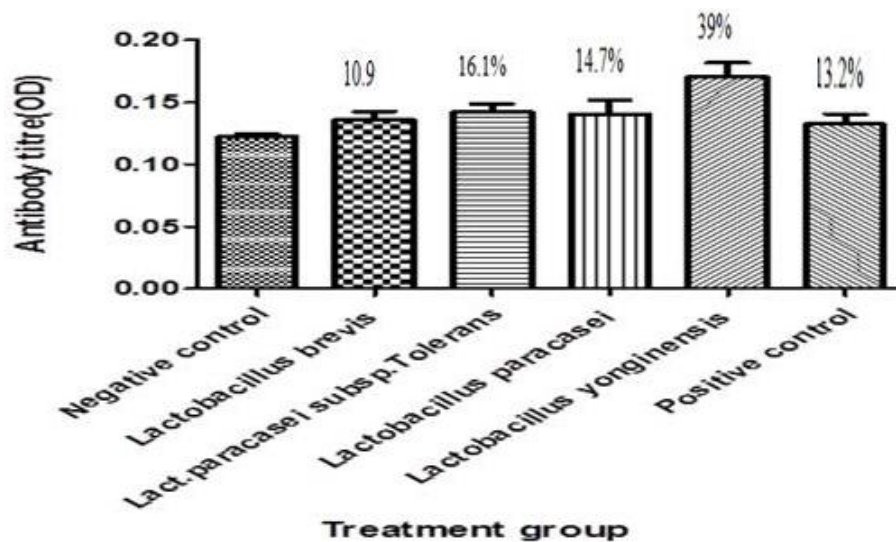
**Figure 1.** The effects of *Lactobacillus* spp on antibody titre (IgG) 4 days post-secondary challenge in mice.



**Figure 2.** The effect of *Lactobacillus* spp on antibody titre (IgG) in albino mice 9 days post-secondary challenge.



**Figure 3.** The effect of *Lactobacillus* spp on antibody titre (IgG1) on albino mice 4 days post –secondary challenge.



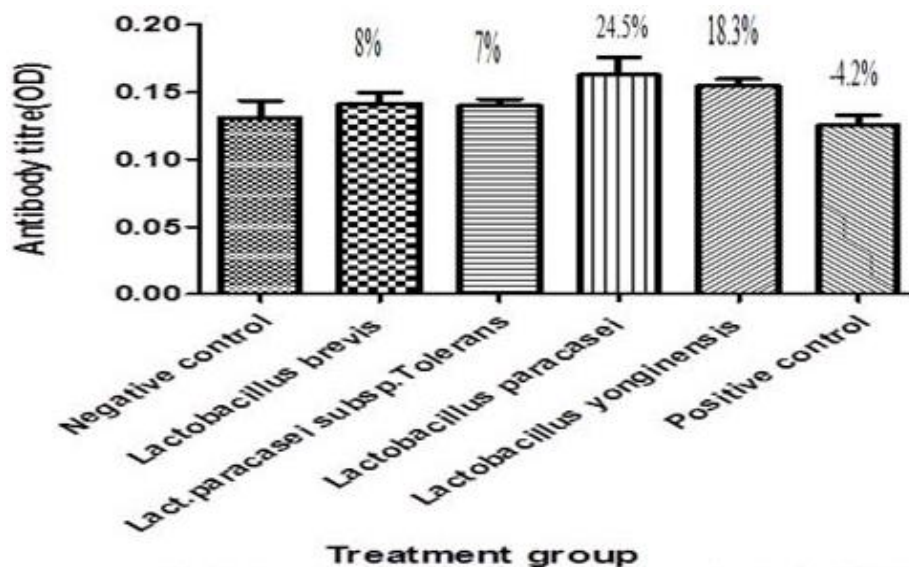
**Figure 4.** The effects of *Lactobacillus* spp. on antibody titre (IgG1) in albino mice 9 days post-secondary challenge.

post-secondary challenge when compared with the negative control. In the case of IgG2a there was no significant percentage stimulation at 4 and only *L. paracasei* produced significant percentage stimulation at 9 days post challenge.

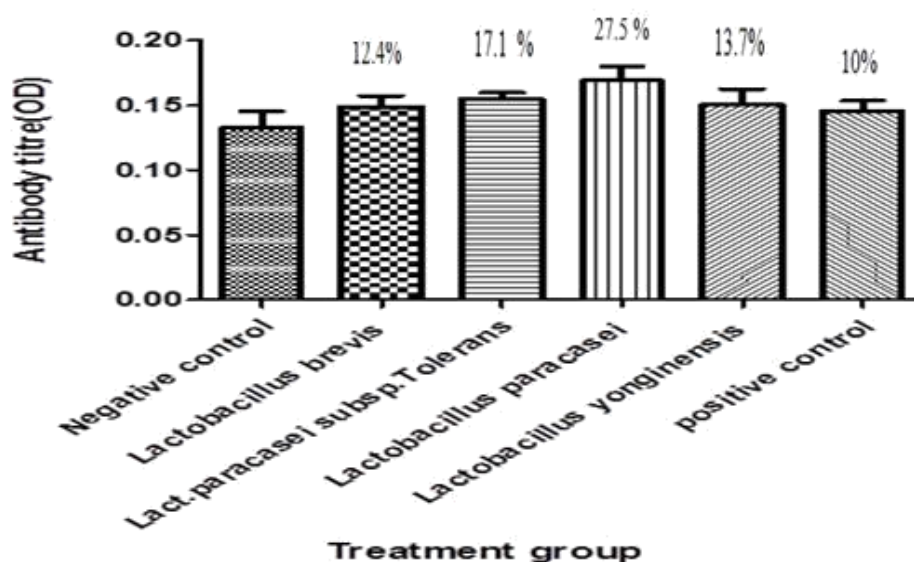
**DISCUSSION**

Humoral immunity refers to antibody-mediated immune responses. Antibodies are produced by plasma cells and protect the host from infection in three main ways: by

binding to pathogens to inhibit their toxic effects or infectivity (neutralization), by coating pathogens and facilitating their uptake and killing by phagocytes (opsonization) and by activating the complement cascade. Immunoglobulin G (Ig) is basically a major component of the circulating immunoglobulin. In mice it is sub classified into IgG1, IgG2a, IgG2b and IgG3 (Pelsue, 2019). The genotypic characterization and blasted sequence result confirmed the presence of *Lb. brevis*, *Lb. paracasei sub. Tolerans*, *Lb. paracasei* and *Lb. yonginensis*. The effects of the different *Lactobacillus* isolates on IgG, IgG1 and IgG2a secretion were



**Figure 5.** The effects of *Lactobacillus* spp on antibody titre (IgG2a) in albino mice 4 days post-secondary challenge.



**Figure 6.** The effects of *Lactobacillus* spp on antibody titre (IgG2a) in albino mice 9 days post-secondary challenge.

investigated at 4 and 9 days post-secondary challenge on albino mice. The serum was analyzed for the antibody titre post-secondary challenge because IgM is the predominant immunoglobulin after initial exposure or post primary challenge. The result of the ELISA showed that there was an insignificant increase in the antibody titre for the different *Lactobacillus* species 4 days post-secondary antigenic challenge for IgG (Figure 1). However at 9 days post-secondary antigenic challenge for IgG, there was marked increase of 100.2, 122.9, 106.4 and 118.3% for

*Lb. brevis*, *Lb. paracasei* sub. *Tolerans*, *Lb. paracasei*, and *Lb. yonginensis*, respectively (Figure 2). The non-significant effect experienced 4 days post challenge could be explained from the fact that the antibody secretion triggered by *Lactobacillus* may be T cell dependent. Antibody production that is T cell dependent involves a cascade of events thus: activated B and T cells remain complexed for 3 days and an exchange of signals, such as CD40/CD40L, leads to initiation of one of two fates for the B cell, some naïve B cells move into the extra

follicular region of the lymph node, where they differentiate into short-lived Plasma cells that produce only IgM as an initial response to infection while others migrate into the follicles where they form germinal centers (GC) (Paus et al., 2006). The GC is the site where B cells undergo affinity maturation, clonal expansion, and ultimately differentiate into high affinity, long lived plasma cells or memory B cells (Jackson and Elsawa, 2015). The sera of the experimental mice analyzed at 4 days post-secondary challenge showed that *Lb. brevis* produced an insignificant effect on IgG1 secretion while *Lb. paracasei sub. Tolersans*, *Lb. paracasei*, and *Lb. yonginensis* produced significant percentage increase as shown in Figure 3. The effect of the isolates on IgG1 9 days post-secondary challenge showed that all the three isolates had insignificant effects on antibody production except *Lb. yonginensis* (Figure 4). Figures 5 and 6 showed that all the isolates produced no significant effect on IgG2a 4 days post-secondary challenge, however only *L. paracasei* produced significant antibody titre 9 days post challenge while the other three produced no significant titre.

The result of this experiment is in agreement with previous studies on the ability of *Lactobacillus* spp to stimulate antibody production (Davras et al., 2018; Easo et al., 2002). A comparison of the effects of the different isolates on the studied immunoglobulins, showed that the isolates produced better percentage stimulation of IgG and IgG1 when compared with the overall effect on IgG2a. This could be because in mice, IgG1 and IgE have been widely used as the surrogate markers of humoral antibody (T-helper [Th2] activation) responses as IL-4 secreted by Th2 cells induces Ig class switching into IgG1 and IgE subclasses (Wang et al., 2007). IL-4 plays an important role in antibody production by inducing the proliferation and differentiation of B cells into plasma cells. On the other hand, IgG2a and IgG3 is the surrogate markers of cellular immune response (Th1 activation) as IFN- $\gamma$  produced by Th1 cells induces Ig class switching into IgG2a or IgG3 subclasses (Stevens et al., 1988).

Palm wine has shown that it is an alcoholic beverage with a difference since it is a natural habitat for immune enhancing, beneficial and generally regarded as safe (GRAS) bacteria, *Lactobacillus*. The four *Lactobacillus* spp were isolated from the fresh and sweet (high sugar content) beverage immediately (0-4 h) after fermentation. Therefore, since fresh palm wine has in abundance these beneficial bacteria, the locals who indulge in excessive consumption of fermented and soured palm wine should be educated on the health benefits of the fresh drink. This will enable them avoid the deleterious effects of alcohol while benefiting maximally from the enormous health benefit of this natural beverage.

## Conclusion

Fresh *E. guineensis* sap (palm wine) is home to *Lactobacillus* spp that has the capacity to positively

stimulate the production of antibodies in mice.

## RECOMMENDATION

The authors are proposing an increased research on this natural beverage especially as it concerns delay/control of fermentation by these beneficial bacteria. This will automatically transform this beverage to a healthier drink than alcoholic.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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