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Original article

Effects of Lactic Acid Bacteria on The Haematological Indices of Carrageenan-Induced Acute Inflammation in Wistar Rats

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Abatract
Immunomodulatory activity of two *Lactobacillus* species isolated from a Nigerian fermented guinea corn slurry product (Ogi) in Wistar rats was studied using paw oedema acute inflammatory model induced by carrageenan. Oedema was induced with 1% Lambda-carrageenan in all experimental groups. Apparently healthy Wistar rats were distributed into five groups (A, B, C, D, and E). Group A received 150 mg of diclofenac sodium treatment following administration of carrageenan. Group B received carrageenan injection only with no treatment. Rats in Groups C and D were treated with 5×10^7 CFU/mL dose of *L. pentosus* 124.2 and *L. plantarum* CIP 103151 while Rats in Groups E were neither administered carrageenan nor treated with LAB. Paw size (mm) was checked at t = 0, 1, 4, 8, 24, 72hrs. ESR, total RBC and WBC was performed on blood samples. Carrageenan induced a very strong inflammatory response in the initial first hour of the experiment which was observed in the changes in rat paw size and ESR measurements. *L. Pentosus* 124.2 and *L. plantarum* CIP 103151 treated groups modulated leukocyte infiltration in the blood circulation of acutely inflamed rats while also maintaining erythrocyte circulation. This study reveals that *L. Pentosus* 124.2 *and L. plantarum* CIP 103151 possesses the ability to play significant immunomodulatory functions.
Keywords: Acute inflammation, *L. plantarum*, *L. pentosus*, leucocytes, carrageenan.

Introduction

The immune system of the body is armed with essential mechanisms for dealing with foreign bodies such as pathogens, damaged cells, stress, some pharmaceuticals or vaccines and toxic compounds (Yoon *et al.*, 2009). Inflammation is a part of the body's immune defense mechanism, where the immune system recognizes and removes harmful stimuli and begins the healing process. It is a very complex response that occurs as a result of an injury, infection or another stimulus, in which several cell types and secreted factors elicits protective immunity, tissue repair and resolution of tissue damage (Shobana, 2017).

'Ogi' is an acid fermented cereal gruel made from maize or corn (*Zea mays*), sorghum (*Sorghum vulgare*), and millet (*Pennisetum americanum*) (Ohenhen and Ikenebomeh, 2007). It is the most popular traditional health-sustaining fermented food in Western Nigeria, and serves as weaning foods. In some communities in South-Western Nigeria, raw 'Ogi' is normally

administered to people suffering from gastroenteritis to reduce or minimize discomforts (Aderiye *et al*., 2007). Lactic acid bacteria (LAB) such as *L. plantarum, L. pentosus and L. fermentum* have been associated with the fermentation of 'Ogi' and have been frequently isolated (Olukoya *et al*., 1994).

Lactobacillus are a member of a broad classification of Lactic acid bacteria (LAB), which are majorly non-pathogenic living microorganisms often consumed with food. This group of bacteria can confer several health benefits when administrated in adequate amounts to the host. Some of the species of *Lactobacillus*, which have been reported to possess immunomodulatory and potential therapeutic properties includes, *L. casei, L. fermentum, L. delbrueckii, L. acidophilus, L. plantarum, and L. reuteri* (de Monero *et al.*, 2011).

Recently many researchers have revealed that certain *Lactobacillus* species have beneficial effects on the immune system (Tsai *et al*., 2012). Several of this LAB species have also been reported to have anti-anemic and anti-oedemic effects which can be either preventive or therapeutic for inflammation, such as metabolic disorders (Tsai *et al*., 2014). A previous study showed that *L. plantarum* OLL2712 possesses strong activity to induce the production of IL-10, an anti-inflammatory cytokine, from bone marrow-derived dendritic cells (DCs) and peritoneal macrophages (Toshimitsu *et al.*, 2016). Understanding the mechanism by which LAB suppresses inflammation is important for the development of food ingredients that have more effective immunomodulatory functions.

Carrageenan-induced inflammatory paw oedema is a classical experimental model for testing and evaluating the anti-inflammatory and immunomodulatory properties of various substances in acute inflammation (Amdekar *et al*., 2012). This study therefore was designed to evaluate the effects *L. pentosus and L. plantarum* isolated from a locally fermented product ('Ogi') in Nigeria for immune regulatory properties using carrageenan induced acute inflammatory model in Wistar rats.

Materials and Methods

Collection of samples

A Nigerian locally fermented guinea corn slurry product ('Ogi') was prepared in aseptic conditions as described by Adebolu *et al.* (2007). The samples were collected in sterile containers and transported immediately to the Department of Microbiology laboratory, Federal University of Technology, Akure for isolation.

Isolation and identification of Bacteria

Lactic acid bacteria present in the fermented guinea corn slurry were isolated on Mann Rogosa Sharpe Agar (MRS) at 37°C in anaerobic condition and identified using standard microbiological methods as described by Bin Masalam *et al.* (2018). Colony morphology of isolates on MRS agar was determined visually. Gram staining reaction was performed to determine the cell morphology as well as catalase test. For molecular identification, cell pellets were harvested from 2 mL of overnight cultures (up to 2×10^9 bacterial cells) of LAB grown in MRS broth. DNA extraction was done using a Jena bioscience DNA purification kit following the manufacturer's instructions. PCR was carried out to amplify nearly the entire region of the 16S rDNA gene. The 16SrRNA gene of the bacteria was amplified using the primer pair 27F-5′-AGAGTTTGATCCTGGCTCAG-3', and 1492R 5'GGTTACCTTGTTACGACTT-3'. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker. They were BLAST-searched to detect similar sequences in the NCBI database (https://www.ncbi.nlm.nih.gov). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair (pairwise deletion option).

Evolutionary analyses were conducted in MEGA X v 10.1.2 (Kumar *et al*., 2018).

Drugs and Chemicals Used

Lambda-carrageenan (CAS 9064-57-7) was purchased from Tokyo Chemical Industry (TCI), diclofenac sodium drug (manufactured by Impulse Pharma PVT. Ltd, Boisar, India. Expiry date; March, 2023) was purchased from a pharmaceutical a government approved shop, Akure, Nigeria.

Evaluation of anti-inflammatory activity

One hundred and five (105) Wistar rats with an average weight of 150g were used for this study. They were housed in stainless steel cages and fed with standard rat chow and water and allowed to acclimatize for one week before the experimental session. All the experimental procedures were carried out following the guidelines of the Institutional Animals Ethics Committee of the Federal University of Technology Akure, Nigeria.

The rats were first divided into 5 groups of 3 rats each (Group A, B, C, D and E). Rats in group A (positive control) were administered carrageenan and treated with diclofenac sodium (150 mg/Kg body weight), Rats in Group B were administered carrageenan only without any treatment (negative control); rats in Groups C and D were administered carrageenan and were treated orally with 5×10^7 CFU/mL of the isolated *L. pentosus* 124.2 *and L. plantarum* CIP 103151 respectively while rats in group E were neither induced nor treated after the development of paw oedema in the rats.

Inflammation was induced using Lambda-carrageenan in the rat's paw tissues. This was done by sub-plantar injection of 1 ml of 1 % Lambda-carrageenan dissolved in sterile saline into the right hind paws of all the rat groups except for the general control group (Group E).

The rats paw size was measured at 20 min before carrageenan injection and after at different time intervals (0, 1, 4, 8, 24, 36, 72, 168, and 336hrs) with an electronic digital vernier caliper and was measured in millimeters (mm) according to the method of Amdekar *et al*. (2012).

Blood sample collection

Blood samples for hematological analysis were collected after each time interval at 0, 1, 4, 8, 24, 36, 72, 168, and 336hrs. The animals were sacrificed with cardiovascular bleeding according to the ethical approval guidelines approved by the Centre for research and development (CERAD) Federal University of Technology Akure, Nigeria; FUTA/ETH/21/06.

Hematological Parameters

Total red blood cell (RBC), white blood cell (WBC) counts and erythrocyte sedimentation rate (ESR) were estimated to monitor inflammation. ESR was estimated by adding blood samples into a Westergren ESR pipette (Citotest Scientific 5830-0002, Jiangsu, China) until the blood level reaches 100 millimeters (mm). The tubes were stored vertically and allowed to sit at room temperature for an hour. The distance between the top of the blood mixture and the top of the sedimentation of RBCs was measured. The ESR (mm) was calculated using the formula: ESR (mm) = Final reading – initial reading as described by Valentini *et al.* (2015).

For the Total Red Blood Cell count, the collected blood was drawn to the 0.5 mark of the standard pipette and mixed with Hayem's solution to fill the pipette tube to the 101 marks. The mixture was transferred into Hemacytometer counting chamber (Neubauer improved), and the total number of RBC was counted under a microscope (x40). The total number of RBCs counted was calculated, using the formula: RBCs = number of cells counted x dilution factor (200) x 1/5 x area factor (0.1mm3). The total circulating WBC count was carried out by mixing 0.02 ml of the collected blood with 0.38ml of Turk's reagent (3% acetic acid with crystal violet dye) in a tube. The mixture was transferred into a counting chamber, and the total number of WBC counted under a microscope (x40). The total number of WBCs counted was calculated, using the formula: WBCs = number of cells counted x depth factor (10) x dilution factor (20) x area factor (0.25).

Statistical Analysis

Data were expressed as the mean ± standard error mean (SEM) calculated over independent time frame of experiments performed in triplicate. One-way analysis of variance (ANOVA) was applied followed by post hoc test; Duncan’s

Comparison Test for difference between treatments mean using SPSS.

Results

Isolation and Identification of *Lactobacillus* species

Two (2) different strains of *Lactobacillus* species were isolated from 'Ogi' confirmed to be *L. pentosus* 124.2 *and L. plantarum* CIP 103151 using conventional and molecular methods as shown in Tables 1-2 and Figures 1-2 below.

Effect of *Lactobacilli* Administration on Carrageenan Induced Inflammation in Rat Paw Tissues

The administration of lactobacilli caused a reduction in the paw oedema size of rats treated with it in a fashion very similar to that of diclofenac. Initially, redness and warmth in the site of injection was noticed followed by the oedema.

Table 1: Biochemical characterization of *Lactobacillus* species isolated from "Ogi"

Isolate	Colonial Morphology	Gram reaction	Catalase	Glucose	Sucrose	Lactose	Probable organism
GCA	Dry, creamy undulating raised surface colony	+ve rods	-	A	A	A	*Lactobacillus* sp.
GCB	White-raised, irregular and smooth colony	+ve rods	-	A	+	+	*Lactobacillus* sp.

KEY: + = Positive, - = negative, A = Acid production, AG= Gas production

Table 2: Molecular Identities of *Lactobacillus* species isolated from 'Ogi'

Isolate code	Biochemical identity	Molecular identity	% similarity	Strain no.	Accession no.
PGC 1	*Lactobacillus* sp.	*L. plantarum*	97.52%	CIP 103151	NR-104573.1
PGC 2	*Lactobacillus* sp.	*L. pentosus*	95.17%	124-2	NR-029133.1

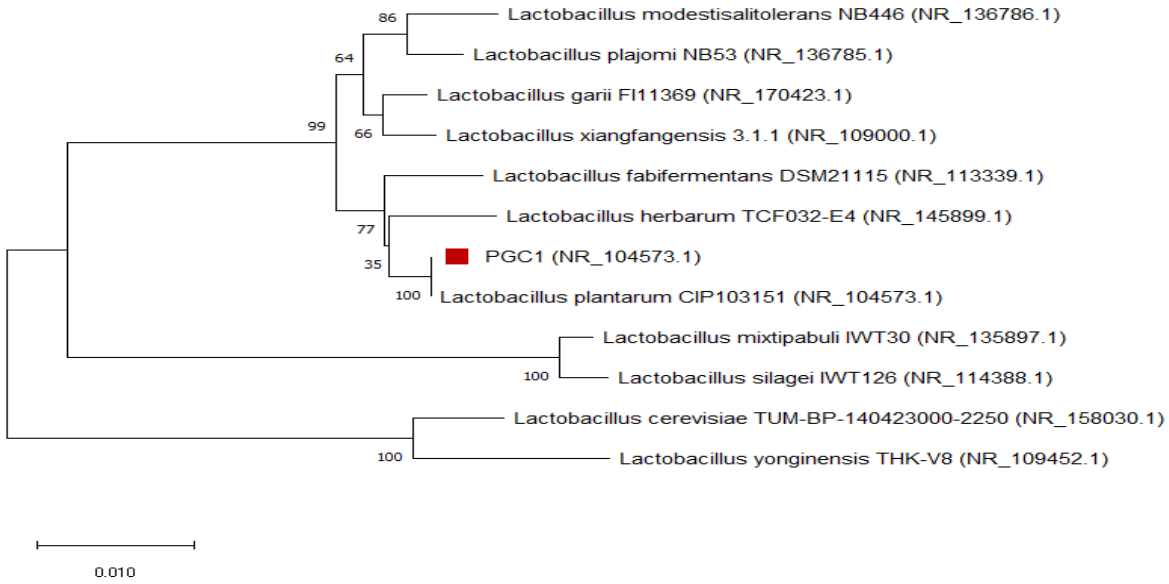

Fig. 1. Phylogenetic analysis of 16S rRNA gene of isolate PGC1 (red) and other related *Lactobacillus spp.* By Neighbor-joining method. Numbers at the nodes indicate the bootstrap support (%) based on 1000 replicates. The scale bar indicates 0.010 nucleotide substitutions per nucleotide position. Genebank accession numbers are given in parenthesis. In the phylogenetic tree, PGC1 (red) and *Lactobacillus plantarum* CIP103151 were clustered together as one clade.

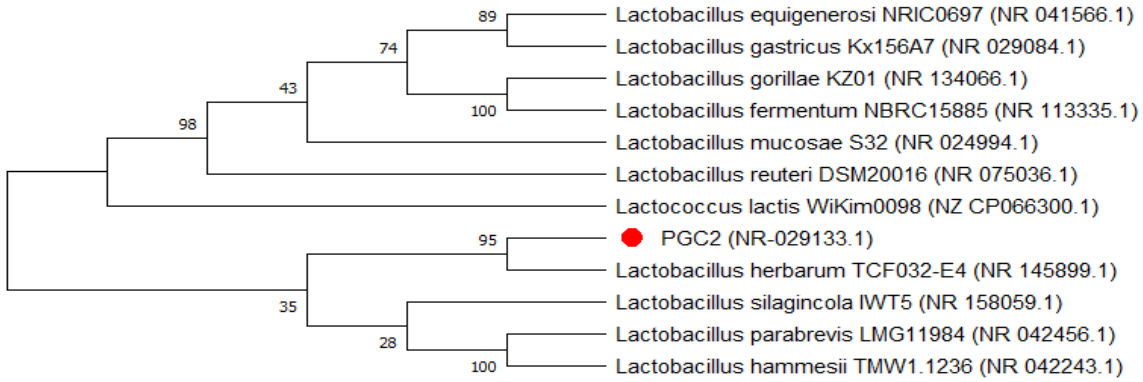

Fig. 2. Phylogenetic analysis of 16S rRNA gene of isolate PGC2 (red) and other related *Lactobacillus spp.* By Neighbor-joining method. Numbers at the nodes indicate the bootstrap support (%) based on 1000 replicates. The scale bar indicates 0.0050 nucleotide substitutions per nucleotide position. Genebank accession numbers are given in parenthesis. In the phylogenetic tree, PGC2 (red) and *Lactobacillus herbarium* TCF032-E4 were clustered together as one clade. PGC2 (red), *Lactobacillus silangicola IWT5*, *parabrevis* LMG11984, *hammesii* TMW1.1236 and *Lactobacillus herbarum* TCF032-E4 also shares similar ancestor.

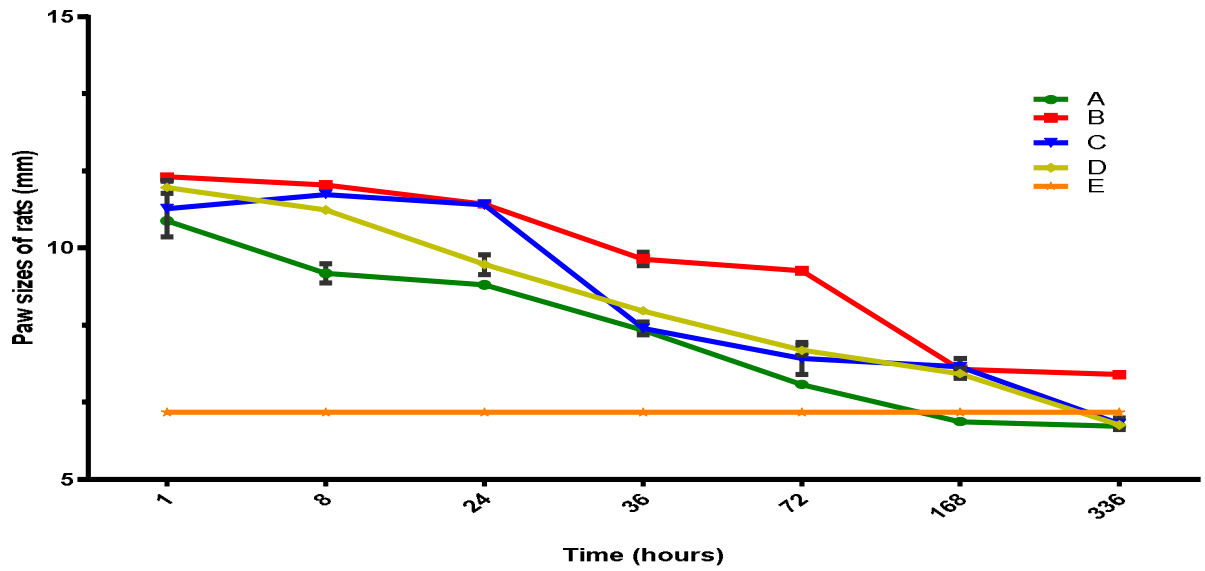

Figure 3: Change in paw size (mm) at t = 0, 1, 4, 8, 24, 72, 168, and 336 hours.
n =3 (significant at P<0.05). Data are expressed as mean ± standard error of two rats per group. Group A: treatment with diclofenac sodium (positive control); Group B: Carrageenan only (negative control); Group C and D: fed with *L. pentosus* 124.2 and *L. plantarum* CIP 103151 respectively, Group E: Carrageenan control.

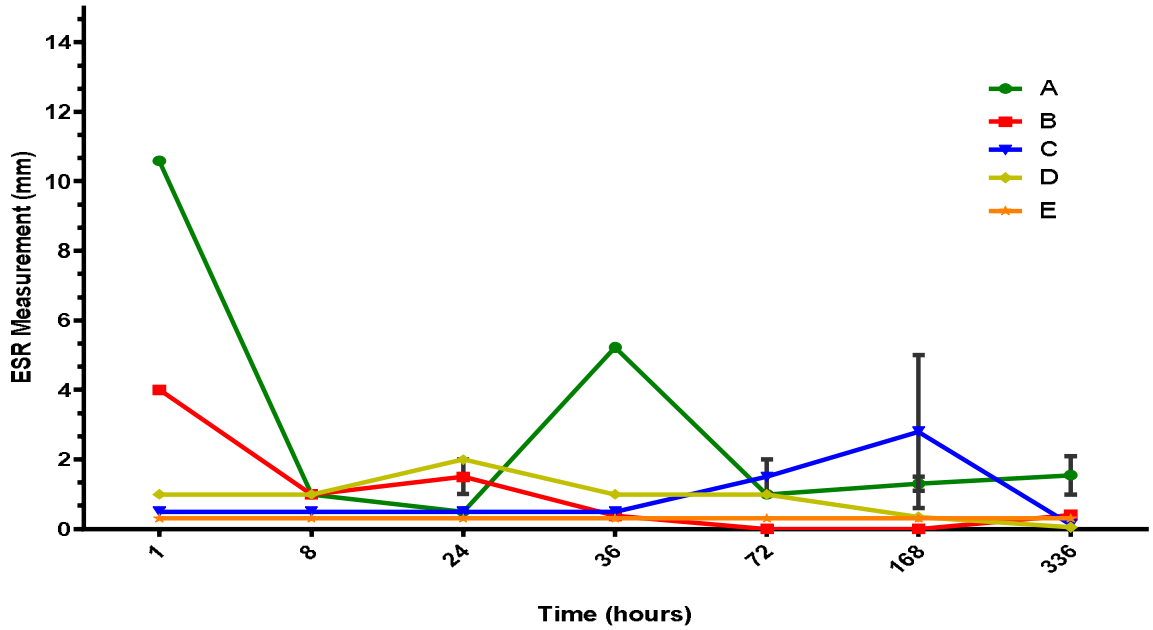

Figure 4: Change in Erythrocyte sedimentation rate
Data are expressed as mean ± standard error of two rats per group. Group A: treatment with diclofenac sodium (positive control); Group B: Carrageenan only (negative control); Group C and D: fed with *L. pentosus* 124.2 and *L. plantarum* CIP 103151 respectively, Group E: Carrageenan control.

Table 3: Effect of oral treatment of LAB on the total red blood cell count (RBC) × $10^6 \mu L^{-1}$ of blood

Groups	Time (h) 1	8	24	36	72	168	336
A	5.68±0.0013	6.23±0.0008	6.58±0.0006^{e}	6.83±0.0001^{f}	6.90±0.0006^{b}	8.20±0.0003^{c}	8.83±0.0004^{C}
B	2.52±0.0008^{c}	6.35±0.0007^{c}	7.33±0.0003^{b}	7.93±0.0002^{a}	6.18±0.0009^{d}	7.53±0.0002^{b}	7.15±0.0000^{b}
C	5.28±0.0008^{d}	6.43±0.0007^{b}	7.10±0.0003^{c}	7.10±0.0000^{e}	7.40±0.0200^{f}	7.50±0.0007^{d}	7.98±0.0001^{f}
D	5.63±0.0000^{b}	6.70±0.0001^{f}	6.98±0.0004^{d}	7.15±0.0003^{c}	7.25±0.0007^{c}	7.78±0.0007^{e}	8.23±0.0005^{d}
E	7.90±0.0002^{a}	7.90±0.0002^{a}	7.90±0.0002^{a}	7.90±0.0002^{a}	7.90±0.0002^{a}	7.90±0.0002^{a}	7.90±0.0002^{a}

Data are expressed as mean ± SEM of three rats per group. Group A: treatment with diclofenac sodium (positive control); Group B: Carrageenan only (negative control); Group C and D: fed with *L. pentosus* 124.2 and *L. plantarum* CIP 103151 respectively, Group E: Carrageenan control.

Groups	Time (h) 1	8	24	36	72	168	336
A	3.62±0.0000^{e}	3.68±0.0500^{e}	4.00±0.0000^{b}	3.87±0.0300^{b}	3.73±0.0500^{d}	4.00±0.0200^{c}	4.10±0.0200^{f}
B	3.40±0.0100^{f}	3.61±0.0000^{f}	9.53±0.0400^{c}	3.71±0.0000^{e}	3.56±0.0700^{f}	10.25±0.0000^{b}	10.95±0.0100^{c}
C	3.68±0.0500^{d}	3.74±0.0500^{d}	3.67±0.0500^{e}	3.76±0.0400^{c}	3.66±0.0500^{e}	3.97±0.0000^{e}	4.35±0.0100^{d}
D	3.88±0.0300^{b}	3.78±0.0300^{b}	9.12±0.0000^{f}	9.29±0.0500^{f}	3.92±0.0300^{b}	3.50±0.0800^{f}	4.56±0.0100^{b}
E	4.26±0.0100^{a}	4.26±0.0100^{a}	4.26±0.0100^{a}	4.26±0.0100^{a}	4.26±0.0100^{a}	4.26±0.0100^{a}	4.26±0.0100^{a}

Data are expressed as mean ± SEM of three rats per group. Group A: treatment with diclofenac sodium (positive control); Group B: Carrageenan only (negative control); Group C and D: fed with *L. pentosus* 124.2 and *L. plantarum* CIP 103151 respectively, Group E: Carrageenan control.

Rats in Group A maintained a constant 6.25±0.00mm all through the experiment which is expected because there was no inflammation induced in them. However, rats in Group E which is the positive control group had a peak at t=0 which was 10.44±0.0058. A much more rapid decrease (when compared with other groups) was also noticed. Groups C and D groups also showed significant decrease in paw volume after the first hour. The values were tested for statistical significance at $p<0.05$ and the effect of treatment means was compared. The results of changes in the paw volume are shown Figure 3.

Effects of Oral Administration of LAB on Erythrocyte Sedimentation Rate

The injection of carrageenan into the right hind paw induced massive increase in the erythrocyte sedimentation rate of the various groups which peaked after the first hour and flattened out for the remaining course of the experiment. In group A rats, ESR at t=0 was 0.30±0.00 mm/hr which remained constant all through the experiment. Group B and E rats showed the most increase in the erythrocyte sedimentation rate after the first hour of injection at 4.0±0.00 significant at $p<0.05$. Group D rats showed the lowest ESR (0.5±0.58 mm/hr) at the first hour. Significant decrease was immediately observed in every group after 8hrs, which remained till the end of 2 weeks (Figure 4).

Effects of Oral Administration of LAB on Red blood cell count (RBC)

Group A rats (positive control) showed a total red blood cell count of 7.90±0.02× 10^6 µL-1 at t= 1 hr which remained the same all through the time course of the experiment. After, the injection of

carrageenan into the right hind paw of other groups, an immediate fall in the total RBC count was observed after the first hour. Group B showed the most decrease shown to 2.52±0.0008c × 10^6 µL-1. Group C (7.10±0.0003x10^6 µL-1) and C (6.98±0.0004×10^6 µL-1) began to indicate increase in the RBC counts at t= 24hours group B showed a continuous low RBC count until after 168hrs. The values were tested for statistical significance at $p<0.05$ and the effect of treatment means was compared (Table 3).

Effects of Oral Administration of LAB on Total white blood cell count (WBC)

Group E rats (carrageenan control) showed a total circulating white blood cell count of 4.26±0.0100 × 10^3 µL-1 at t= 1hr which remained the same all through the time course of the experiment. Injection of carrageenan into the right hind paw of other rat groups triggered a progressively massive influx of leucocytes at t= 24 hours at 9.53±0.0400. *L. plantarum* CIP 103151 treated group showed a major control of the influx of WBC at 24 hours and 36 hours (3.67±0.0500 x 10^3 µL-1 and 3.76±0.0400 x10^3 µL-1) respectively as shown in Table 4.

Table 4: Effect of oral treatment of LAB on the total white blood cell count (WBC) × 10^3 μL^{-1} of blood

Discussion

Lactobacillus sp. isolated from 'Ogi' may be as a result of the fermentation process involved in its production which supports enzymatic activities and its growth. This assumption is related to the findings of Orji *et al*. (2020). *L. plantarum* CIP 103151 and *L. pentosus* 124-2 have been reported by several researchers to be present in several cereal fermentations, such of that is the findings of Olatunde *et al*. (2018) in which they molecularly identified potential probiotic lactic acid bacteria in effluents generated during 'Ogi' production.

The treatment of the induced male Wistar rats with LAB may account for the decrease in the inflammation of the rats' paws after two weeks of inducement. Such findings have been reported by Sarika *et al.* (2012) in which they examined the anti-inflammatory activity of *Lactobacillus* on Carrageenan-induced paw edema in male Wistar rats. In this model of inflammation, *L. plantarum* CIP 103151 and *L. pentosus* 124-2 had very consistent anti-inflammatory activity and showed significant decrease in paw size of the rats (Group C - E). The significant decrease in paw volume of rats in the test groups after treatment with *Lactobacillus* sp indicates *Lactobacillus* sp has an effective anti-inflammatory agent. Some researchers have also reported the anti-inflammatory property of LAB in paw edema (Sarika *et al*., 2012; Archer *et al*., 2015). The cause of acute inflammation is biphasic. First phase starts with the release of histamine, serotonin, and kinins after the injection of phlogistic agent in the first few hours (Bhukya *et al*., 2009). While the second phase is related to the release of prostaglandins like substances in 2-3 hours. Second phase is sensitive to both the clinically useful steroidal and nonsteroidal anti-inflammatory agent (Sarika *et al.,* 2012). Prostaglandins are mainly responsible for acute inflammation. *Lactobacillus* sp might be containing some anti-inflammatory agent which is responsible for the blockage of prostaglandins and inflammatory pathway (Sarika *et al*., 2012).

The decrease in the total RBC count of experimental rats after the first hour of carrageenan inducement may be as result of immune response to hyperalgesia and inflammation caused by carrageenan, causing platelet depletion which in turn abrogates the hyperalgesia. This assumption is related to the findings of Yamashita *et al*. (2011) in which they reported platelet depletion abrogates hyperalgesia induced by *Bothrops jararaca* venom and carrageenan. The increase in the erythrocyte counts at 36hours and 8hours respectively after treatment with LAB and decrease in erythrocyte until after 168hrs of untreated group is an indication that LAB had significant effect on RBC of the experimental rats. Carrageenan induced a disease state of increase in total circulating white blood cells of the experimental rats after 24 hours of injection, which was more and quickly resolved in groups treated with LAB strains. This is similar to the findings of Cicala *et al*. (2008) in which they also reported Carrageenan to induce increase in total white blood cells of experimental rats in their study.

The modulation of leukocyte infiltration may be associated with the release of specific substances or proteins which are responsible for modulating the response of various immune cells (lymphocytes, granulocytes, macrophages, mast cells) (Abdul-Kalam, 2018). Modulation of inflammatory mediators by *L. pentosus* and *L. plantarum* could be another mechanism for regulating inflammatory diseases.

Conclusion

This present study shows that *L. pentosus* 124.2 *and L. plantarum* CIP 103151 possess immunomodulatory properties while *L. plantarum* CIP 103151 is the most effective in restoring balance to the circulating immune cells and suppresses the effect of inflammation in acutely inflamed rats. Consumption of 'Ogi' might serve as a conventional way of alleviating inflammation in the absence of commercial drugs.

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