Journal of Applied Biological Sciences
E-ISSN: 2146-0108 17(1): 163-172, 2023
DOI: https://doi.org/10.71336/jabs.1078
Research Article

NEUTROPHIL-INFILTRATING REGULATORY POTENTIAL OF *Lactobacillus pentosus* 124-2 IN ACUTE INFLAMMATORY MODEL

Babayemi Olawale Oladejo

Federal University of Technology, Department of Microbiology, Akure, Nigeria

**Corresponding Author:*
E-mail: booladejo@futa.edu.ng

(Received 17th May 2022; accepted 28th November 2022)

ABSTRACT. Neutrophils are often responsible for pathological inflammation, as well as other immunological responses. Controlling their activity is crucial to maintaining host integrity, particularly preventing against chronic inflammation. Inflammatory oedema was induced in paw of male Wistar rats using 1% iota-carrageenan. Control group was neither induced nor treated with lactic acid bacteria (LAB), while negative control group was induced without treatment. Experimental group was orogastrically treated with *Lactobacillus pentosus* 124-2 ($5x10^7$ CFU/mL), while positive control group received diclofenac sodium treatment (150 mg/kg body weight) following carrageenan administration. Biochemical assays including myeloperoxidase (MPO), malondialdehyde (MDA), and nitric oxide (NO) were performed on the blood samples at t=1, 4, 24, and 72 hours, and tissue histology was done using standard methods to monitor neutrophil infiltration. LAB treated group showed marked decrease in NO and MPO activity at 4 hours following inducement as well as increase in MDA activity till 72 hours ($p<0.05$). Neutrophil infiltration was markedly controlled in the LAB treated group between 4 to 72 hours and compared favorably with the diclofenac sodium. This inflammatory model established that administered *L. pentosus* 124-2 inhibited neutrophil infiltration and activation resulting in the significant MPO reduction, while also regulating NO production.

Keywords: *Neutrophils, inflammation, lactobacillus, carrageenan, oedema.*

INTRODUCTION

Neutrophils, also called polymorphonuclear (PMN) leukocytes, are the most common immune cells and have long been thought to represent the first layer of protection in the immune system's innate arm. They are critical in host defense against infection including those caused by fungi and bacteria [1]. They generate reactive oxygen species (ROS) with strong microbicidal activity [2]. Indeed, the functions of neutrophils in homeostasis as well as pathological inflammation and immunological responses have rekindled interest in neutrophil biology. They catch and eliminate invading microbes by the release of granules, phagocytosis and intracellular destruction and the development of neutrophil extracellular traps (NETs) basically after identifying the pathogens [1]. Sequel to this, the delayed phase of chemically-induced acute inflammation as a result of carrageenan treatment involves the production of nitric oxide (NO), neutrophil-derived free radicals, and NETs such myeloperoxidase (MPO) [3]. MPO and NO are already key active indicators of diseases linked with neutrophil inflammatory activity [4]. These reactive

species derived from MPO serve an important role in human defense as well as neutrophil antimicrobial activity against a variety of illnesses, principally by phagocytosis. When neutrophils are activated, lysosomes fuse with phagosomes, resulting in the release of MPO.

Recently, many researchers have revealed that certain *Lactobacillu*s species have beneficial effects on the immune system [5]. Moreover, these *Lactobacillu*s species have also been reported to have neutrophilic modulatory functions which can be preventive or therapeutic for inflammation [6], including *L. acidophilus, L. casei, L. fermentum, L. plantarum, L. pentosus,* and *L. reuteri,* which have been shown mostly using animal models [7].

Here in this study, acute inflammation was induced in the paw of Wistar rats using iota carrageenan, which has been documented to be an effective experimental model for the evaluation of inflammatory drugs, such as assessing the neutrophilic activity of an indigenously derived *L. pentosus* 124-2.

MATERIALS AND METHODS

Drugs and Chemicals

Iota-carrageenan (CAS 9064-57-7) was purchased from Tokyo Chemical Industry (TCI), Japan, while diclofenac sodium (Impulse Pharma PVT. Ltd, Boisar, India; Expiry date March, 2023) was purchased from a government approved pharmaceutical outlet in Akure, Nigeria.

Preparation of L. pentosus strain

L. pentosus 124-2 with accession number NR-029133.1 was previously isolated and reported by Oladejo and Adiji [8] from fermented guinea corn slurry, which is a popular breakfast cereal and weaning food consumed in South West Nigeria. The bacterium was isolated and identified using the methods described by Bin Masalam *et al*. [9] and Oladejo and Oluwasola [10].

Evaluation of anti-inflammatory activity

Ninety-six (96) male Wistar rats with an average weight of 150 g were grouped and housed in stainless steel cages. They were fed with water and standard commercial feed diet purchased from a certified Veterinary Clinic in Akure, Nigeria (feed composition: Hydrolyzed maize starch (65%), fish meal (22%), palm oil (5%), bone meal (2.5%), salt (0.50%) and cellulose (5%) and acclimatized for one week before the experimental session. All experimental techniques were conducted in accordance with the Institutional Animals Ethics Committee guidelines of the Federal University of Technology Akure, Nigeria (FUTA/ETH/21/06). Inflammation was induced in all rat groups except the Group A which is the general control group *via* sub-plantar injection of 1% iota-carrageenan (1 mL) dissolved in sterile saline into the right hind paws. Afterwards, the rats were separated into four groups (n = 6). Group B received no LAB treatment (negative control), while Group C were orally administered with 5×10^7 CFU/mL of *L. pentosus* 124-2 treatment. Group D were subjected to diclofenac sodium treatment (positive control; 150 mg/kg body weight). Following the formation of oedema in the paw of the rats, sub-plantar tissues were then removed from the paws for histological and biochemical

analysis. The rat paw thickness was monitored at different time intervals with a digital Vernier caliper.

Histopathological evaluation

The rat paw tissues were collected and fixed in 10% neutral buffered formalin before being processed using conventional tissue processing procedures. The stained slides were photomicrographed using a digital microscope (OMAX microscopes, Irvine, CA, USA) for histomorphometry cell count. The National Institutes of Health-sponsored Image Analysis and Processing for Java (Image J) program was used to count the neutrophils using x400 magnification (USA).

Inflammatory biochemical assays

The sub-plantar tissues were immediately separated from the rats at the designated time of the experiment [11]; followed by homogenizing in eight volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% potassium chloride and centrifuged for 15 minutes at 4 ^{o}C using 10000 x g speed. The supernatants were collected and used to estimate the total protein levels. The protein content was estimated according to Lowry *et al*. [12] using bovine serum albumin as a reference standard.

Myeloperoxidase (MPO)

The amount of neutrophil infiltration/activation was determined using MPO activity. To generate an o-dianisidine solution, o-dianisidine dihydrochloride (o-dianisidine 16.7 mg) was first dissolved in 90 mL distilled water and 10 mL potassium phosphate buffer. For every test, this solution was newly produced. The o-dianisidine mixture received 50 µL of diluted hydrogen peroxide (H_2O_2) (4 µL of 30 percent H_2O_2 diluted in 96 liters of distilled water). Each well received 70 µL of the tissue homogenate and 2000 µL of o-dianisidine mixture containing hydrogen peroxide. A spectrophotometer was used to read the absorbance at 450 nm.

Nitric oxide (NO)

Griess reagent was prepared by adding 0.1% N-(1-Naphthyl) ethylenediamine (NED) and 1% sulfamic acid in a 5% phosphoric acid solution. A 96 well microtiter plate was filled with 50 µL of the sample and 100 µL of Griess reagent. To allow for color development, the mixture was incubated at room temperature for 10 minutes without exposure to light. The absorbance was measured at 550 nm in a microplate reader within 30 minutes.

Malondialdehyde (MDA)

An aliquot of 0.4 mL of the material was combined with 1.6 mL of Tris-KCl buffer and 0.5 mL of 30% TCA. Thereafter, 0.5 ml of 0.75% TBA was added and placed in an 80 ^{o}C water bath for 45 minutes. The mixture was chilled in ice before being centrifuged at 3000 g for 15 minutes. The absorbance of the clear supernatant was measured at 532 nm in comparison to a distilled water reference blank. The MDA level was calculated using a standard method described by Adam-Vizi and Seregi [13]. Lipid peroxidation was calculated in /mg protein or gram tissue units using a molar extinction coefficient of $1.56x10^5$ M-1Cm-1.

$$\text{MDA (units/mg protein)} = \frac{\text{Absorbance x volume of mixture}}{E_{532nm} \text{ x volume of sample x mg protein}}$$

Statistical Analysis

Quantitative data were expressed as the mean±standard error mean (SEM) of experiments performed in n=6. Descriptive analysis for two-way analysis of variance (ANOVA) was applied followed by post hoc test and Tukey's Multiple Comparison Test for difference between the treatments mean using GraphPad Prism version 8.0.

RESULTS AND DISCUSSION

Nitric oxide (NO) activity of L. pentosus 124-2 in acute inflammatory model

The carrageenan injection caused a significant increase in NO activity in paw of rats immediately after an hour of the injection as shown in Fig. 1. The activity of NO in the rat paw tissues mediated by the administration of *L. pentosus* 124-2 showed marked decrease after 4 hours, which was highly similar ($p<0.05$) to the carrageenan only (untreated) and drug treated (positive control) rat groups. The decline in NO activity was consistent and continuous till the end of 72 hours ($p<0.05$).

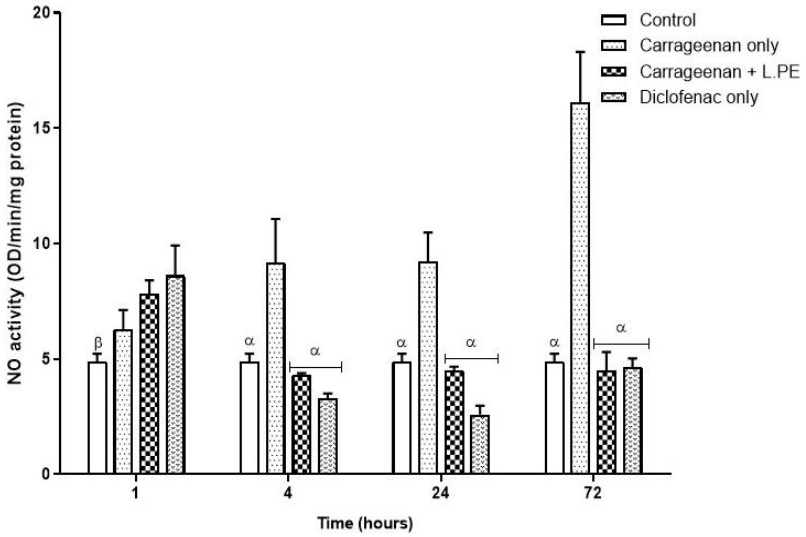

Fig. 1. *Effect of L. pentosus 124-2 administration on Nitric oxide (NO) activity in rat paw tissues at various intervals. Data are expressed as mean±standard error of 6 rats per group (n=6). Values were found statistically significant at $p<0.05$. α; indicates a significant difference as compared to the negative control (carrageenan only) group, while β indicates a significant difference as compared to the positive control (diclofenac) group.*

MPO activity of L. pentosus 124-2 in acute inflammatory model

The injection of carrageenan in the untreated rat group caused a significant increase in MPO activity in paw of rats. However, oral administration with *L. pentosus* 124-2 remarkably decreased the MPO activity when compared to the carrageenan only and

diclofenac treated group (positive control) ($p<0.05$). The suppression was observed to have similar mark to the general control rat groups, which were neither injected nor treated and the trend continued through the four selected significant hours as shown in Fig. 2.

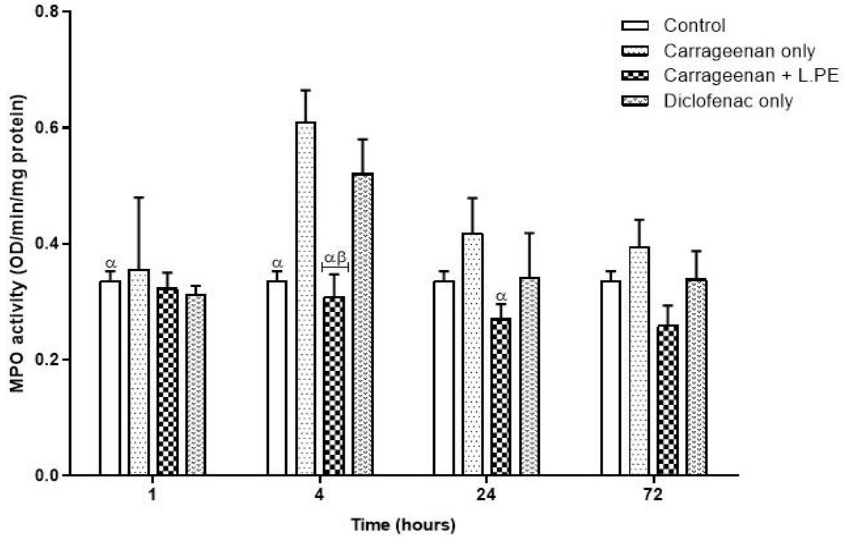

Fig. 2. *Effect of L. pentosus 124-2 administration on MPO activity in rat paw tissues at various intervals. Data are expressed as mean±standard error of 6 rats per group (n=6). Values were found statistically significant at $p<0.05$. α; indicates a significant difference as compared to the negative control (carrageenan only) group, while β indicates a significant difference as compared to the positive control (diclofenac) group.*

Malondialdehyde (MDA) L. pentosus 124-2 in acute inflammatory model

Malondialdehyde activity of the treated inflamed rats showed that the oral administration of *L. pentosus* 124-2 remarkably increased the accumulation of MDA in rat paw (also significant against diclofenac sodium treatment $p<0.05$), indicating the accumulation of these peroxide in the rat paw tissues as shown in Fig. 3.

Infiltrating neutrophil cells mediated activity of L. pentosus 124-2 in acute inflammatory model

The neutrophil cell count to the rat paws showed that treatment with *L. pentosus* 124-2 rapidly and continuously decreased infiltrations of neutrophils, particularly from 4 hours till 72 hours, and was significant (*α;* $p<0.05$) compared with the carrageenan control group (i.e., rats without injection or treatment). The diclofenac sodium treated group (positive control) however, had sustained increase in neutrophil influx till 72 hours, which was similar to rats in the negative control group; rats without treatment as shown in Fig. 4.

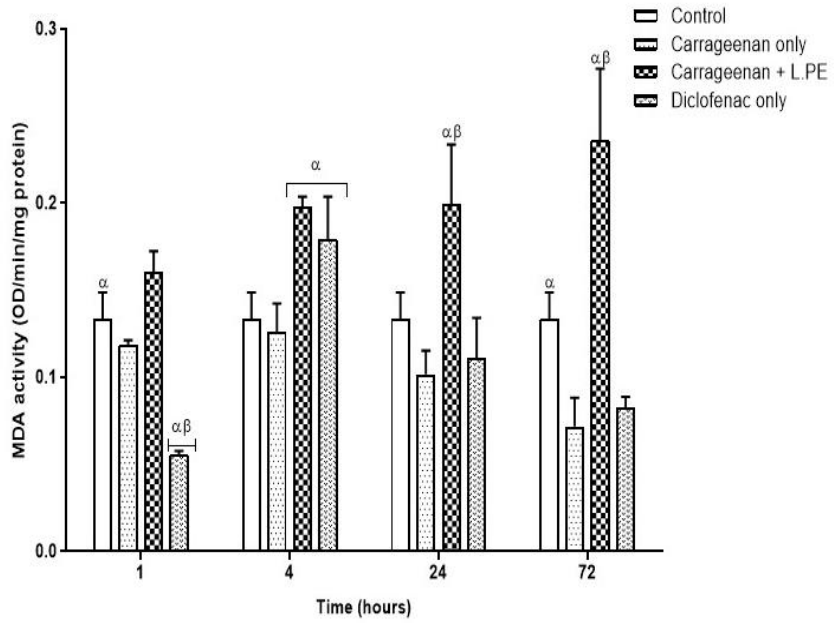

Fig. 3. *Effect of L. pentosus 124-2 administration on malondialdehyde activity in rat paw tissues at various intervals. Data are expressed as mean±standard error of 6 rats per group (n=6). Values were found statistically significant at p<0.05. α; indicates a significant difference as compared to the negative control (carrageenan only) group, while β indicates a significant difference as compared to the positive control (diclofenac) group.*

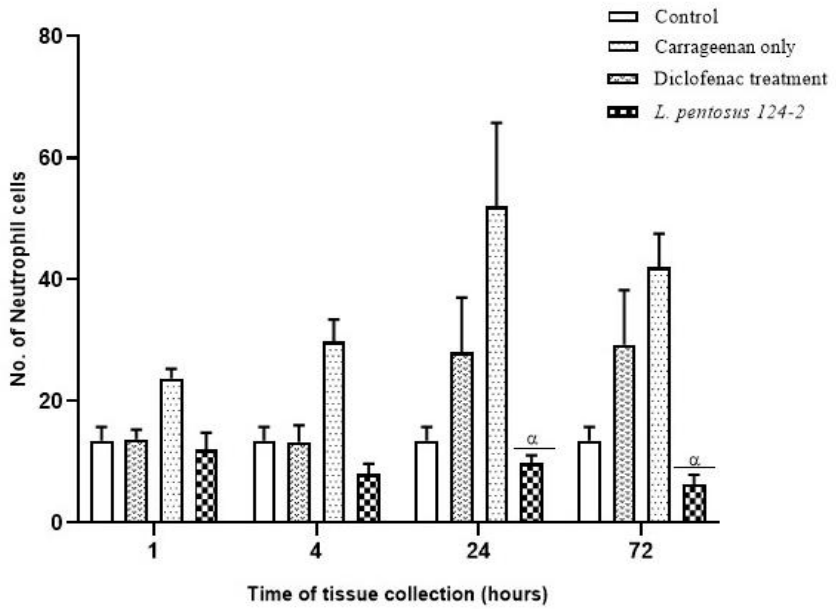

Fig. 4*. Cell count of infiltrating neutrophils in the paw tissue of rats treated L. pentosus 124-2 at the various time frames. α indicates a significant difference (p<0.05) as compared to the negative control (carrageenan only) group.*

Histopathological evaluation of neutrophil mediated activity of L. pentosus 124-2 in acute inflammatory model

Infiltration of immune cells was initially observed at a significant number in the LAB treated groups as at 4 hours in contrast to a very low number of infiltrating cells observed at the first hour in all groups. At 24 hours, when a massive influx of infiltrating neutrophils was observed in the untreated rat group and diclofenac sodium treatment group, LAB treated rat group had lesser visible number of infiltrating cells, despite the presence of slightly more infiltrating cells in the diclofenac sodium treatment rat group (Fig. 5). At 72 hours there was a side effect of the diclofenac treatment with stains of red blood cells observed in the tissue of rat paw.

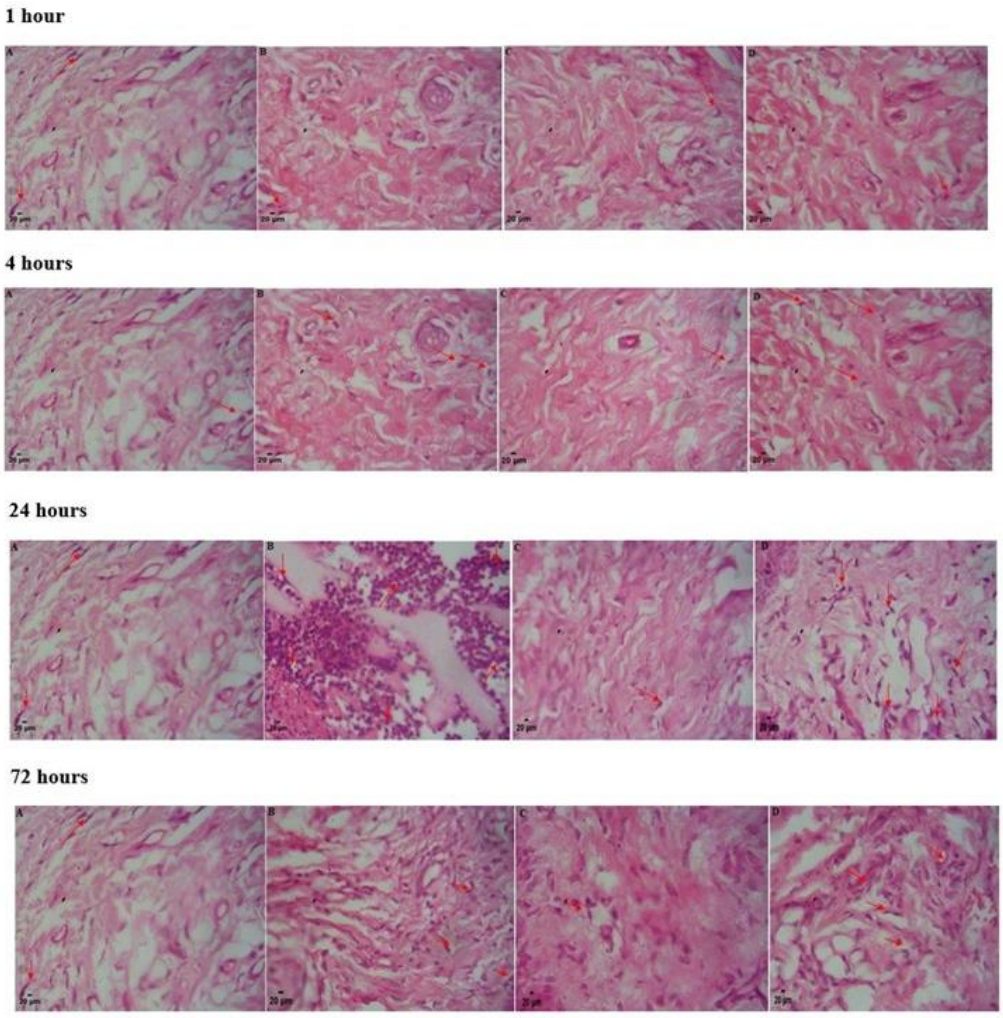

Fig. 5. *Photomicrographs of the histopathological features analysis of paw tissue from rats treated with L. pentosus 124-2 at the various time intervals. Keys: (A) General control (B) Negative control (C) L. pentosus 124-2 (D) Positive control. Red arrows: Infiltrating cells*

Carrageenan-induced acute inflammation normally takes place in two phases (biphasic) with the release of certain inflammatory mediators/chemicals such as

histamine, kinins, serotonin, e.t.c. The release of these chemicals pave the way for the accumulation of fluids in the endothelial tissues, whereas the delayed phase of the inflammatory response as a result of carrageenan treatment (after 1-2 hrs) is associated with neutrophil infiltration [2, 14]. This is followed by the activation of pattern recognition receptor (PRRs) such as Toll like receptors in an inflammatory cascade series [15, 16].

This delayed phase of acute inflammation specifically involves the generation of neutrophil-derived free radicals, nitric oxide (NO), and pro-inflammatory cytokines [3]. During inflammation, the recruitment of neutrophils to the site is often an important source of NO [17], in which this study shows a massive influx of neutrophils in the histopathological cell counts and image following carrageenan-induced inflammation, and a corresponding increase in the level of NO. However, a significant reduction by *L. pentosus* 124-2 treatment was observed in this study. It is possible that this reduced level of NO could result from decrease or alteration in neutrophil mobilization and activity in the paw tissue of rats, which is essential for resolution of acute inflammation from becoming chronic. Increased neutrophils in lung tissues have been linked with patients suffering from inflammatory lung diseases as well as animal models of oxidant lung injury with clear evidences showing that its accumulation (neutrophils) in the lungs thus play a key role in pulmonary failure caused by chronic inflammation [18]. *L. pentosus* 124-2 therefore might provide a new ground for regulating neutrophils influx in inflammation as seen in this study.

Furthermore, during inflammation, on neutrophil activation, there are series of interrelated and interdependent events from the generation of neutrophil-derived nitric oxide (NO), fusion of lysosomes with phagosomes results in release of NETs such as myeloperoxidase enzyme (MPO). Myeloperoxidase can react rapidly with nitric oxide and peroxynitrite so that during inflammation, there is a strong possibility that these reactions will have an impact on oxidative damage caused by neutrophils [19]. Also, activation of polymorphonuclear neutrophils (PMNs) with concomitant release of MPO is thought to be regulated in a nitric oxide–dependent manner [20]. Increased MPO levels not only relates with NO activity, but is a major marker of neutrophil activity and active inflammatory disease state. Elevated concentrations of circulating MPO have been reported to be linked with the presence of coronary artery disease and predict cardiovascular events in patients suffering from acute coronary syndrome [21]. In this study, MPO levels were significantly raised as was observed in all rat groups injected with carrageenan, only with the exception of treatments with *L. pentosus* 124-2 which was in fact better than the standard drug used in this study as positive control. This suggests that the administered *L. pentosus* 124-2 inhibited neutrophil infiltration and activation resulting in the significant MPO reduction, while also regulating NO production which explains possible *L. pentosus* 124-2's anti-neutrophilic activation potential.

CONCLUSION

This study shows that *L. pentosus* 124-2 possess protective effect against excessive neutrophil activity and oxidative damage products production occasionally associated with acute inflammation. Consumption of fermented foods containing indigenous lactic acid bacteria such as *L. pentosus* isolated from fermented guinea corn slurry in this study

is encouraged as a conventional treatment of acute inflammatory reactions in the absence of commercial drugs.

Acknowledgement. The technical support provided by the Departments of Microbiology and Human Anatomy, Federal University of Technology, Akure, Nigeria is appreciated.

Conflict of Interest. The author declared that there is no conflict of interest.

Authorship Contributions. Concept, Design, Data Collection or Processing: Analysis or Interpretation, Literature Search, Writing: B.O.O.

Financial Disclosure. This research received no grant from any funding agency/sector.

REFERENCES

[1] Rosales, C. (2018): Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? Frontiers of Physiology9:113. doi: 10.3389/fphys.2018.00113

[2] Mansouri, M.T., Hemmati, A.A., Naghizadeh, B., Mard, S.A., Rezaie, A., Ghorbanzadeh, B. (2015): A study of the mechanisms underlying the anti-inflammatory effect of ellagic acid in carrageenan-induced paw edema in rats. Indian Journal of Pharmacology 47:292. https://doi.org/10.4103/0253-7613.157127

[3] Halici, Z., Dengiz, G.O., Odabasoglu, F., Suleyman, H., Cadirci, E., Halici, M. (2007): Amiodarone has anti-inflammatory and anti-oxidative properties: an experimental study in rats with carrageenan-induced paw edema. European Journal of Pharmacology 566:215 221. https://doi.org/10.1016/j.ejphar.2007.03.046

[4] Adeboboye, C.F., Oladejo, B.O., Adebolu, T.T. (2022): Effects of *Lactobacillus fermentum* on inflammatory-associated anaemia and leucocyte homeostasis in carrageenan-induced paw oedema in Wistar rats. Bacteria Empire 5: 1, e327. https://doi.org/10.36547/be.327

[5] Tsai, Y. T., Cheng, P. C., Pan, T. M. (2012): The immunomodulatory effects of lactic acid bacteria for improving immune functions and benefits. Applied Microbiology and Biotechnology 96(4), 853–862. https://doi.org/10.1007/s00253-012-4407-3

[6] Tsai, C. C., Lin, P. P., Hsieh, Y. M., Zhang, Z. Y., Wu, H. C., Huang, C. C. (2014): Cholesterol-lowering potentials of lactic acid bacteria based on bile-salt hydrolase activity and effect of potent strains on cholesterol metabolism *in vitro* and *in vivo*. The Scientific World Journal 2014, 690752. https://doi.org/10.1155/2014/690752

[7] De Moreno De Leblanc, A., Del Carmen, S., Zurita-Turk, M., Santos Rocha, C., Van De Guchte, M., Azevedo, V., Miyoshi, A., Leblanc, J.G. (2011): Importance of IL-10 Modulation by Probiotic Microorganisms in Gastrointestinal Inflammatory Diseases. ISRN Gastroenterology, 2011, pp.1–11. https://doi.org/10.5402/2011/892971

[8] Oladejo, B.O., Adiji, P.I. (2021): Effects of Lactic Acid Bacteria on the Haematological Indices of Carrageenan-Induced Acute Inflammation in Wistar Rats. FUTA Journal of Life Sciences 1(1): 61-70.

[9] Bin Masalam, M.S., Bahieldin, A., Alharbi, M.G., Al-Masaudi, S., Al-Jaouni, S.K., Harakeh, S.M, et al (2018): Isolation, Molecular Characterization and Probiotic Potential of Lactic Acid Bacteria in Saudi Raw and Fermented Milk. Evidence-Based Complem Alt Med. pp.1–12. https://doi.org/10.1155/2018/7970463

[10] Oladejo, B.O., Oluwasola, H.M. (2021): Oral Administration of *Weissella* and *Pediococcus* sp Alleviates Formalin Induced Inflammation in Rats by Cytokine Modulation. Ife Journal of Science 23(2): 1-10. https://dx.doi.org/10.4314/ijs.v23i2.10

[11] Chiu, K., Lau, W.M., Lau, H.T., So, K.F., Chang, R.C. (2007): Micro-dissection of Rat Brain for RNA or Protein Extraction from Specific Brain Region JoVE (7):269. https://doi.org/10.3791/269

[12] Lowry, O., Rosebrough, N., Farr, A., Randall, R. (1951): Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193:265. https://doi.org/10.1016/S0021-9258(19)52451-6

[13] Vizi, V., Seregi, M. (1982): Receptor Dependent Stimulatory Effect of Noradrenaline on Na+/K+ ATPase in Rat Brain Homogenate. Role of Lipid Peroxidation. Biochemical Pharmacology 31, 2231-2236. http://dx.doi.org/10.1016/0006-2952(82)90106-X

[14] Gilligan, J.P., Lovato, S.J., Erion, M.D., Jeng, A.Y. (1994): Modulation of carrageenan-induced hind paw edema by substance P. Inflammation 18:285-292. https://doi.org/10.1007/BF01534269

[15] Amdekar, S., Roy, P., Singh, V., Kumar, A., Singh, R., Sharma, P. (2012): Anti-inflammatory activity of *lactobacillus* on carrageenan-induced paw edema in male wista rats. International Journal of Inflammations 75.pp6. https://doi.org/10.1155/2012/752015

[16] Silver, F.R.F., Dore, C.M., Marques, C.T., Nascimento, M.S., Benevides, N.M.B, Rocha, H.A, Chavante, S.F., Leite, E.L. (2010): Anticoagulant activity, paw edema and pleurisy induced carrageenan: action of major types of commercial carrageenan. Carbohydr. Polym. 79: 26–33. https://doi.org/10.1016/j.carbpol.2009.07.010

[17] Alabi, A.O, Ajayi, A.M., Omorogbe, O., Umukoro, S. (2019): Anti-nociceptive and anti-inflammatory effects of an aqueous extract of blended leaves of *Ocimum gratissimum* and Psidium guajava Clinical Phytoscience 5(34):1-9. https://doi.org/10.1186/s40816-019-0130-2

[18] Aratani, Y. (2018): Myeloperoxidase: Its role for host defense, inflammation, and neutrophil function. Archives of Biochemistry and Biophysics 640: 47-52. https://doi.org/10.1016/j.abb.2018.01.004

[19] Von Leitner, E.C., Klinke, A., Atzler, D., Slocum, J.L., Lund, N., Kielstein, J.T., Maas, R., Schmidt-Haupt, R., et al. (2011): Pathogenic cycle between the endogenous nitric oxide synthase inhibitor asymmetrical dimethylarginine and the leukocyte-derived hemoprotein myeloperoxidase. Circulation 124(24):2735-45. doi: 10.1161/CIRCULATIONAHA.111.060541.

[20] Kettle, A. J., Winterbourn, C. C. (1997): Myeloperoxidase: a key regulator of neutrophil oxidant production. Redox Rep. 3(1):3-15. doi: 10.1080/13510002.1997.11747085.

[21] Winterbourn, C.C., Kettle, A.J., Hampton, M.B. (2016): Reactive oxygen species and neutrophil function. Annual Review of Biochemistry 85:765-792. https://doi.org/10.1146/annurev-biochem-060815-014442