

## ORAL ADMINISTRATION OF *Weissella* AND *Pediococcus* sp ALLEVIATES FORMALIN INDUCED INFLAMMATION IN RATS BY CYTOKINE MODULATION

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### ABSTRACT

This study was designed to study the cytokine modulatory activity of three lactic acid bacteria (LAB) strains, *Weissella cibaria* II-1-59, *Weissella confusa* JMC 1093, and *Pediococcus pentosaceus* DSM20336 isolated from a Nigerian locally fermented food condiment; "iru" using paw oedema acute inflammatory model induced with 1% formalin in Wistar rats. Rats were distributed into six groups (A-F). Rats in Groups A were neither administered formalin nor treated with LAB, while Group B received formalin injection only. Rats in Groups C, D, and E were administered formalin and were treated orally with  $2 \times 10^7$  CFU/ml of *Weissella cibaria* II-1-59, *Weissella confusa* JMC 1093, and *Pediococcus pentosaceus* DSM20336 respectively, while Group F received diclofenac sodium treatment following administration of formalin. The dose of LAB strain used for the oral treatment was  $2 \times 10^7$  CFU/ml for all the groups while the dose of diclofenac sodium used was 150 mg/kg body weight of the rats. Paw thickness (mm) was checked at  $t = 0, 1, 4, 8, 24, 72, 168$  and 336 h. Cytokine assay for C-reactive protein (CRP), Interleukin (IL-10) and Transforming growth factor (TGF- $\beta$ ) was performed on serum samples of the rats using Enzyme-linked immunosorbent assay (ELISA). Oral administration of *W. cibaria* II-1-59 showed the best significant decrease in the paw thickness of the rats, which was followed by *P. pentosaceus* DSM20336 and *W. confusa* JMC 1093 respectively, and was shown to be statistically significant at  $P < 0.05$ . There was also a significant decrease (below standard 2000 pg/ml) in the secretion of pro-inflammatory biomarker (CRP) in all LAB treated groups at 1 hour, while there was an increase in the serum levels of anti-inflammatory cytokines IL-10 and TGF- $\beta$  in Groups C-E rats which was maximally increased in *W. confusa* JMC 1093 treated rats. This study suggests that *W. cibaria* II-1-59, *W. confusa* JMC 1093 and *P. pentosaceus* DSM20336 possess anti-inflammatory potentials.

**Keywords:** Inflammation, Cytokines, *Weissella*, Formalin, Oedema, *Pediococcus*

### INTRODUCTION

The immune system of the body recognizes and removes harmful stimuli in a bid to initiate the healing process or recovery from variety of factors such as pathogens, damaged cells, stress, and irritation which triggers a series of inflammatory responses (Michels *et al.*, 2019). Inflammation can be induced as a result of trauma, microbial invasion, or noxious compounds and can later result to tissue damage. It starts rapidly, becomes severe in a short time and symptoms may last for a few days (Jain *et al.*, 2015).

Cytokines are important regulators of inflammatory responses in the body. They are pleiotropic proteins or small glycoproteins produced by a number of cell types, such as leukocytes which regulate immunity, inflammation and hematopoiesis (Gulati *et al.*, 2016). Cytokines are secreted proteins that have a specific effect on the interactions and communications between cells. They include interleukins, chemokines, interferons, and tumor necrosis factors, which have a multitude of

pleiotropic effects in various organs (Monastero and Pentyala, 2017). Cytokines involved in inflammatory responses are basically of two types: pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines act in concert to generate inflammation in host defense and disease, but anti-inflammatory cytokines attenuate inflammation and induce repair (Gulati *et al.*, 2016).

There are various components to an inflammatory reaction injury; such as oedema formation, leukocyte infiltration and granuloma. Oedema formation in the paw is the result of a synergism between various inflammatory mediators that increases vascular permeability and blood flow (John and Shobana, 2012). Although some acceptable results have been achieved with the use of conventional drugs like non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and herbal supplements for the treatment of inflammation, yet a life-threatening condition of chronic diseases that presents a major threat to the health and longevity of individuals still exists

(Barcelos *et al.*, 2019). Hence, there is a need for an alternative source of treatment.

A fermented locust bean ('Iru') is a very good source of a number of microbial strains, especially from the genera of *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Bacillus* (Afolabi *et al.*, 2016). Lactic acid bacteria (LAB) are also known as probiotics and can act as immune-modulators (Pessione, 2012). These organisms confer health benefit on the host when administered in sufficient amount. Formalin is a strong chemical used to induce acute inflammation. Formalin induced paw oedema in rat is one of the most suitable tests to screen for inflammatory responses.

Therefore, this study aims to evaluate the anti-inflammatory properties and cytokine mediated immune-regulating activities of lactic acid bacteria isolated from indigenous fermented locust beans ('Iru') using Wistar rats induced with formalin.

## MATERIALS AND METHODS

### Collection of Samples

Fermented locust bean ('Iru') was purchased from Oba market, Akure, Ondo State, southwest, Nigeria. The samples were put in a sterile polythene bag and transported immediately to the Department of Microbiology Laboratory, Federal University of Technology, Akure for bacteriological analysis.

### Isolation and Identification of Bacteria

The types of bacteria present in the fermented locust bean ('Iru') were isolated and identified using standard microbiological methods as described by Bin Masalam *et al.*, (2018). Molecular identification of the isolated LAB was carried out by harvesting cell pellets from 2 ml of overnight cultures (about  $2 \times 10^9$  bacterial cells) of LAB grown in De Mann Rogosa and Sharpe Agar (MRS) broth and DNA extraction was done using a JenaBioscience DNA purification kit following the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out to amplify the 16srRNA gene of the bacteria 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 80 V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. A 100 bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker. For the sequencing, PCR products were purified with Exo sap and then subjected to DNA Sanger sequencing after which the resulting data was analyzed by ABI Sequencing Analysis software (version 5.2). They were then BLAST-searched to detect similar sequences in the NCBI database.

### Drugs and Reagents

Formalin, diclofenac sodium (Impulse Pharma Pvt. Ltd, Boisar, India.), and cytokines ELISA assay kits; CR-P, IL-10 and TGF-beta (MELSIN medical co. Limited, Jilin province, China) were purchased for the assay.

### Evaluation of Anti-inflammatory Activity

Healthy male Wistar rats with an average weight of 150 g were used for this present study. The rats were housed in stainless steel cages. They were fed with standard commercial rat feed and water and allowed to acclimatize for one week before the experimental session. For the anti-inflammatory assay, inflammation was first induced by sub-plantar injection of 0.1 ml of 1% freshly prepared formalin into the right hind paws of all the rats except the rats in general control group (Group A). After inflammation had been induced, which was observed physically by the swelling and redness of the paw as seen in paw volume measurements, rats were divided into 5 groups (Groups B - F) of 3 rats each. Rats in Group B were administered formalin only without any treatment (negative control); rats in Group C, D, and E were administered formalin and were treated orally with  $2 \times 10^7$  CFU/ml of *W. cibaria* II-1-59, *W. confusa* JMC 1093, and *P. pentosaceus* DSM20336 respectively while rats in Group F (positive control) were administered formalin and treated with diclofenac sodium (150 mg/Kg body weight) after the development of paw oedema in the rats. Changes in rat paw thickness was measured at 20 min before injection at 0 hr and after LAB treatment following the sub-plantar injection of formalin at different time intervals (1, 4, 8, 24, 72, 168 and 336 h) with a digital vernier caliper measured in millimeters (mm) (Amdekar *et al.*, 2012). All the experimental procedures were

carried out following the guidelines of the Institutional Animals Ethics Committee of the Federal University of Technology Akure, Nigeria.

### Blood Sample Collection

At different intervals earlier mentioned, the rats were bled through cardio puncture according to the guidelines of Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA). Blood samples collected were subjected to cytokine ELISA assays. Blood samples for the cytokine assay were allowed to coagulate for 60 minutes at room temperature and then centrifuged at 1500 g for 15 minutes and crude serum was kept in new cryovial tubes. The harvested serum was stored at  $-20^{\circ}\text{C}$  until use (Amdekar *et al.*, 2012).

### Cytokine Assay

C-reactive protein (CRP) levels (Pro-inflammatory biomarker) in nanogram per millilitre (ng/ml) for acute phase proteins produced in the liver, IL-10 and TGF- $\beta$  (anti-inflammatory cytokines) in picogram per millilitre (pg/ml) were estimated with an ELISA analyzer

reader (Labtech Auto Elisa P, Mumbai, India). Serum samples were used to perform the assay according to the method of Valentini *et al.* (2015).

### Statistical Analysis

Data were expressed as the mean  $\pm$  standard error of 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. Bonferroni's Multiple Comparison Test for difference between treatments groups compared with control (Group B) was done using GraphPad Prism version 5.0.

## RESULTS

### A. Isolation and Identification of *Lactobacillus* sp

Table 1 shows the three (3) LAB strains that were isolated from 'Iru' sample, The BLAST search of Genbank for all the LAB isolates provided the percentage similarity between the bacteria tested and those detected in Genbank. The 16srRNA sequence analysis identified the isolates to be *Weissella cibaria* II-1-59, *Weissella confusa* JMC 1093, and *Pediococcus pentosaceus* DSM20336.

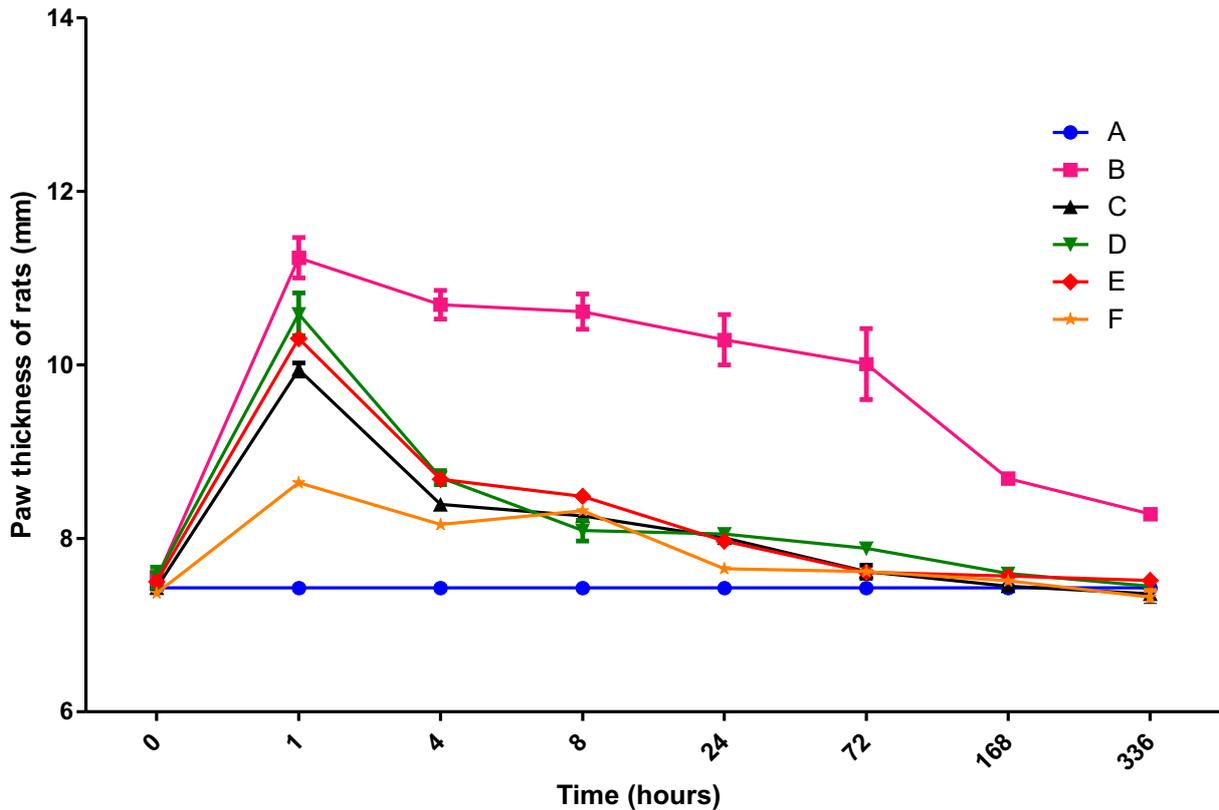
**Table 1:** Molecular Identities of Lactic acid bacteria (LAB) Isolated from Fermented Locust Bean ('Iru')

Isolate code	Biochemical identity	Molecular identity	% similarity	Strain no.	Accession no.
HBL1	<i>Lactobacillus</i> sp.	<i>Weissella cibaria</i>	97.27%	II-1-59	NR_036924.1
HBL2	<i>Lactobacillus</i> sp.	<i>Weissella confusa</i>	96.77%	JMC 1093	NR_040816.1
HBL3	<i>Pediococcus</i> sp.	<i>Pediococcus pentosaceus</i>	95.84%	DSM20336	NR_042058.1

### B. Effects of *Weissella* sp and *Pediococcus* in Formalin Induced Inflammation in Rat Paw Tissues

Figure 1 shows changes in paw thickness (mm) of rats before and during LAB treatments. At 1 hour post sub-plantar injection of 0.1 ml of 1% solution of formalin, the highest paw thickness was recorded in formalin inflamed Wistar rats. The paw thickness of rats in group A (Normal control) at 0 hour was  $7.43 \pm 0.00$  mm which remained constant throughout the period of the experiment. Group B rats showed the most increase in the paw thickness after the first hour of

injection, which was significant at  $p < 0.05$ . Significant decrease was only observed in this group after 72 hrs, which was gradual till the end of 336 hrs. In Group C, D and E (LAB treated Groups), significant decrease in paw volume was observed after 4 hrs of treatment till the end of the experiment. Group F (Diclofenac treated group) showed the least increase in paw volume after the first hour when compared with other treatment groups. The values were tested for statistical significance at  $p < 0.05$  and the effect of treatment means was compared.



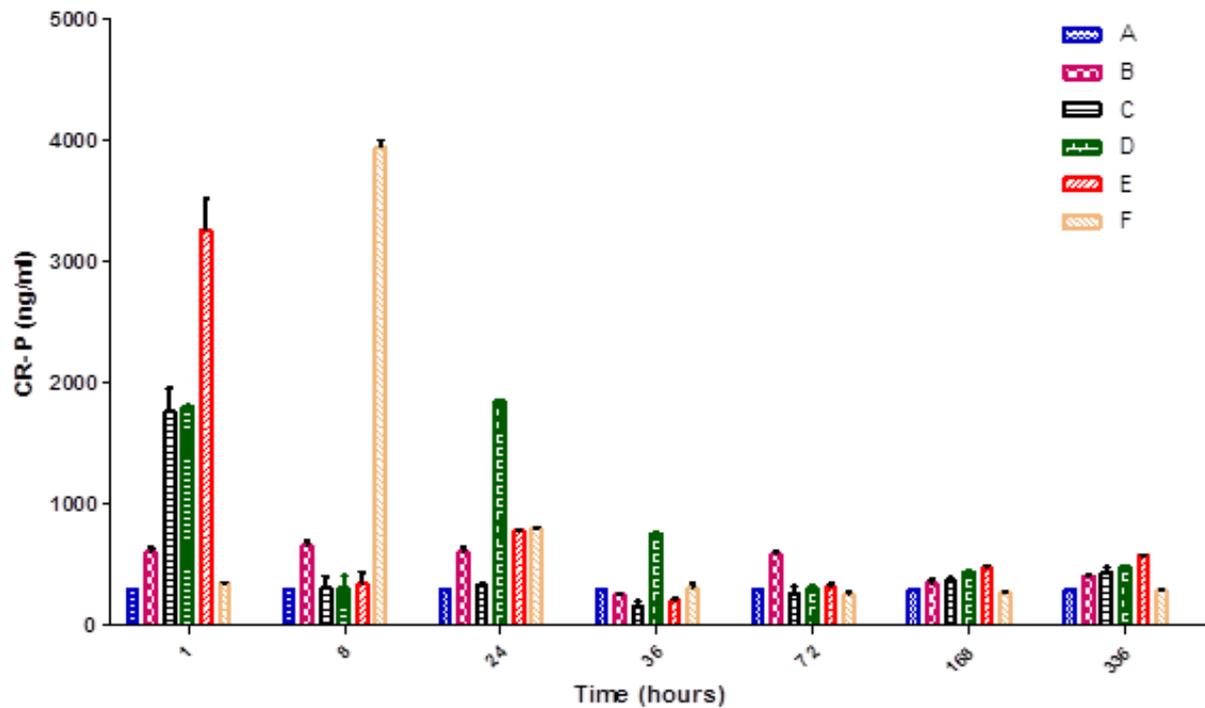
**Figure 1:** Change in Paw Thickness (mm) of Rats before and during LAB Treatment at t = 0, 1, 4, 8, 24, 72, 168, and 336 hrs

n=3 (significant at P<0.05). Data are expressed as mean ± standard error of three rats per group. Group A: neither induced nor treated; Group B: Formalin only (negative control); Group C: treated with *W. cibaria* II-1-59; Group D: treated with *W. confusa* JMC 1093; Group E: treated with *P. pentosaceus* DSM20336; Group F: treatment with diclofenac sodium (positive control). Values were found statistically significant at P<0.05.

**C. Pro-inflammatory Biomarker (CRP)**

Figure 2 reveals the secretion of CRP in all the groups of the rats within the first hour of the experiment which was induced by the injection of formalin into the right hind paw of the rats. In Group A, serum CRP levels was shown to be 300.00±0.00 ng/ml which remained constant throughout the period of the experiment. The highest CRP concentration was observed in

Group F rats after 8 hours (3943.00±0.00 ng/ml) and followed by Group E rats at the first hour (3261.00±0.00 ng/ml). Additionally, the LAB treated groups showed significant reduction in the CRP levels after 24 hrs till the end of 336 hrs. Also, significant reduction in the CRP levels was observed in Group F after 36 hours compared with other treatment groups



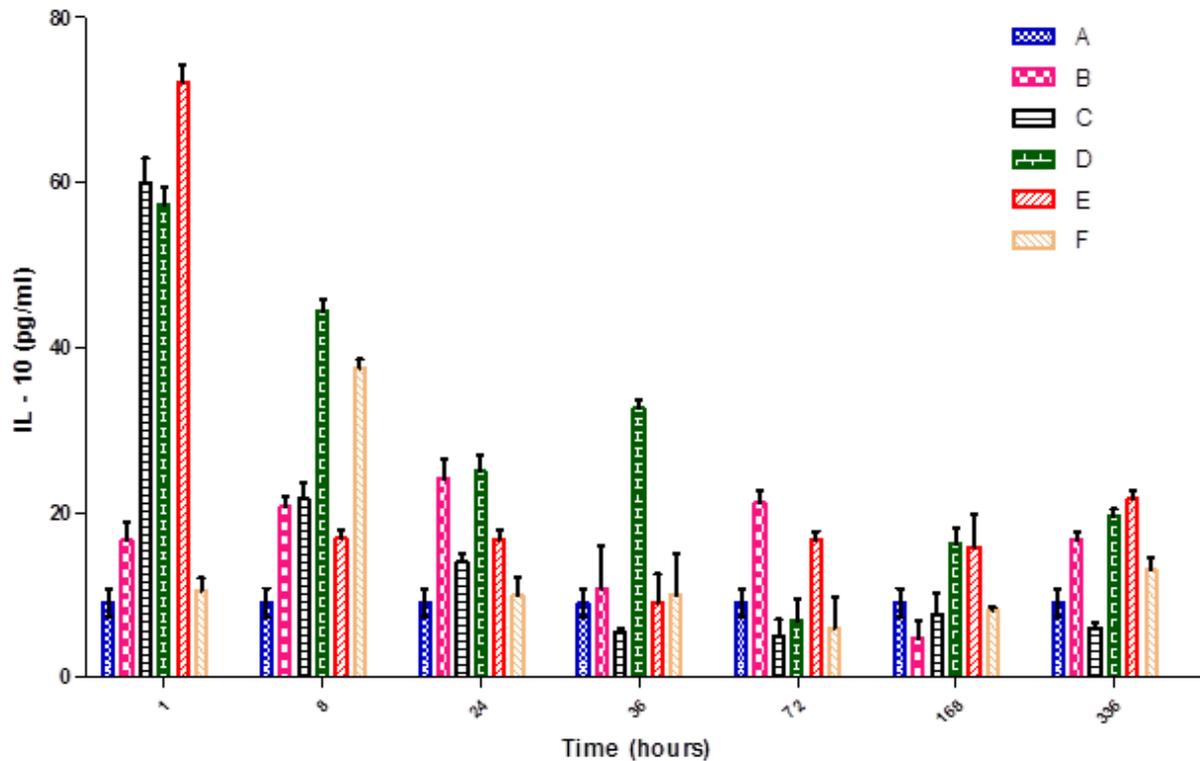
**Figure 2:** Production of CRP (ng/ml) at  $t = 1, 8, 24, 36, 72, 168$  and  $336$  hrs

$n = 3$  (significant at  $P < 0.05$ ). Data are expressed as mean  $\pm$  standard error of three rats per group. Group A: neither induced nor treated; Group B: Formalin only (negative control); Group C: treated with *W. cibaria* II-1-59; Group D: treated with *W. confusa* JMC 1093; Group E: treated with *P. pentosaceus* DSM20336; Group F: treatment with diclofenac sodium (positive control). Values were found statistically significant at  $P < 0.05$ .

#### D. Anti-inflammatory Cytokine (IL-10)

Figure 3 depicts that the serum level of IL-10 in Group A at the first hour was  $9.00 \pm 0.00$  pg/ml and which remained constant throughout the period of the experiment. In untreated Group B rats, relatively low production of IL-10 was observed. An increased production of IL-10 was observed in LAB treated Groups reaching its maximum level at 1 hour. IL-10 level was highest in Group E rats ( $72.20 \pm 0.00$  pg/ml) followed by Groups C and D at  $59.9 \pm 0.00$  pg/ml and

$57.3 \pm 0.00$  pg/ml, respectively while Group F showed the lowest value of IL-10 ( $10.50 \pm 0.00$  pg/ml) among treated rat groups. Group F also showed an increase in the production of IL-10 ( $37.50 \pm 0.00$  pg/ml) at 8 hrs. Groups D and E rats showed serum IL-10 level between the ranges of  $6.90 \pm 2.52$  pg/ml -  $32.00 \pm 0.00$  pg/ml after 24 hours till the end of 336 hrs. The values were tested for statistical significance at  $p < 0.05$  and the effect of treatment means was compared.



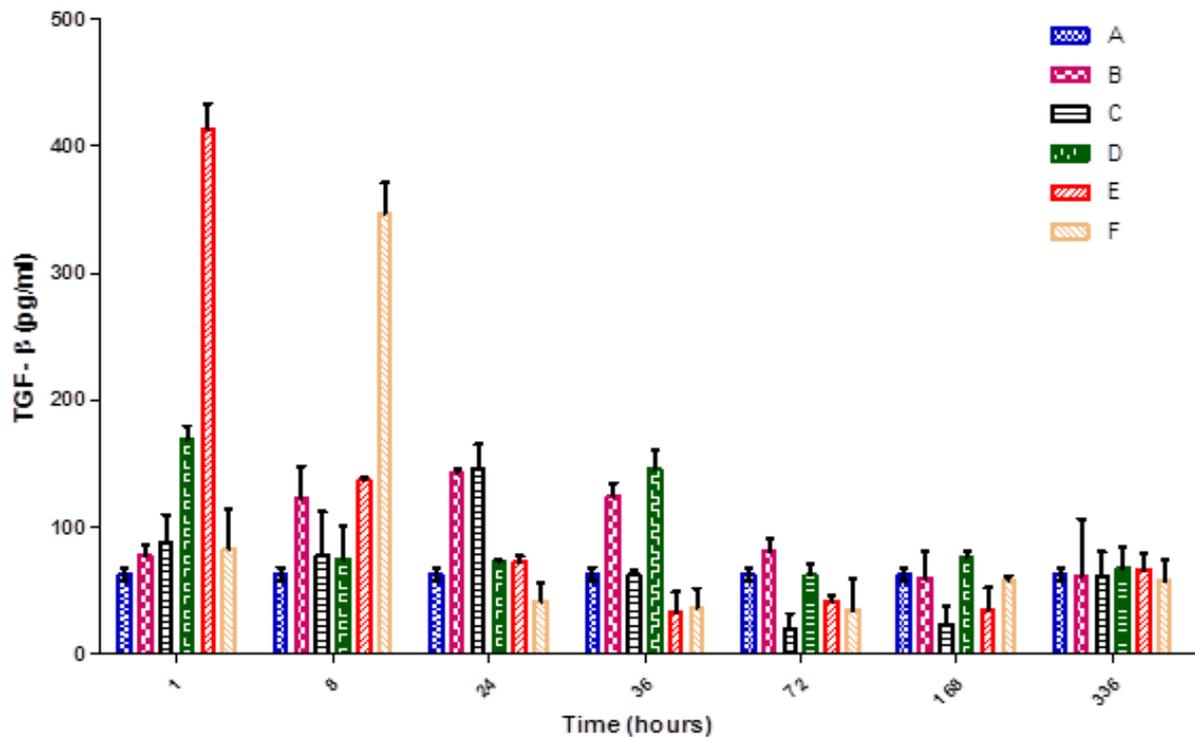
**Figure 3:** Production of IL-10 (pg/ml) at  $t = 1, 8, 24, 36, 72, 168$  and  $336$  hrs

$n = 3$  (significant at  $P < 0.05$ ). Data are expressed as mean  $\pm$  standard error of three rats per group. Group A: neither induced nor treated; Group B: Formalin only (negative control); Group C: treated with *W. cibaria* II-1-59; Group D: treated with *W. confusa* JMC 1093; Group E: treated with *P. pentosaceus* DSM20336; Group F: treatment with diclofenac sodium (positive control). Values were found statistically significant at  $P < 0.05$ .

#### E. Anti-inflammatory Cytokine (TGF- $\beta$ )

Figure 4 reveals that the serum TGF- $\beta$  level of Group A rats at  $t = 1$  hour was  $62.90 \pm 5.00$  pg/ml and remained constant till the end of 336 hrs. An increase in the production of TGF- $\beta$  was observed in Group B after the first hour and continued till the end of 72 hours. The highest

value for TGF- $\beta$  was observed in Group E ( $413.10 \pm 0.00$  pg/ml) at  $t = 1$  hr and followed by Group F ( $347.00 \pm 0.00$  pg/ml) at 8 hours while the lowest value was observed in Group C ( $19.7 \pm 12.03$  pg/ml) at 72 hours. The values were tested for statistical significance at  $p < 0.05$  and the effect of treatment means was compared.



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n = 5 (significant at  $P < 0.05$ ). Data are expressed as mean  $\pm$  standard error of three rats per group. Group A: neither induced nor treated; Group B: Formalin only (negative control); Group C: treated with *W. cibaria* II-1-59; Group D: treated with *W. confusa* JMC 1093; Group E: treated with *P. pentosaceus* DSM20336; Group F: treatment with diclofenac sodium (positive control). Values were found statistically significant at  $P < 0.05$ .

## DISCUSSION

Many lactic acid bacteria (LAB), such as *Lactobacillus* sp., *Leuconostoc* sp., *Pediococcus* sp. and *Weissella* sp. contribute to the fermentation of food products (Ouoba *et al.*, 2010) including African locust beans (*Parkia biglobosa*). In this study, LAB isolated from African locust beans (Iru) were identified as *W. cibaria* II-1-59, *W. confusa* JMC 1093 and *P. pentosaceus* DSM20336 with accession no NR\_036924.1, NR\_040816.1 and NR\_042058.1 respectively. Although *Bacillus subtilis* has been reported as the dominant bacterium involved in the fermentation, however, recent studies have also implicated lactic acid bacteria (LAB) in the alkaline fermentation of locust beans (Ouoba *et al.*, 2010, Ajayi, 2014, Afolabi *et al.* 2016). This result appears similar to other studies that have successfully isolated and identified different types of lactic acid bacteria from fermented food products including African locust beans (Uaboi-Egbenni *et al.*, 2009, Ouoba *et al.*, 2010, Afolabi *et al.* 2016). *Weissella* sp., a relatively recent member of the lactic acid bacteria family, similar to the *Leuconostoc* was first identified in 1993 (Ahn *et al.*, 2013; Fessard and Remize,

2017). In particular, *W. cibaria* has been isolated from fermented foods such as Korean kimchi, Greek salami, and Spanish sausages (Kwak *et al.*, 2014). *Weissella cibaria* has been shown to possess anti-cancer, anti-inflammatory, antibacterial, anti-fungal, and immune boosting potentials (Kwak *et al.*, 2014).

At the injection of a compound like formalin, release of inflammatory mediators such as histamine, serotonin, kinins, etc., starts the first phase leading to accumulation of fluids in the endothelial tissues. Within few hours, the second phase takes off with further release of prostaglandins and related substances. This is also characterized by leukocyte infiltration (especially primary response from neutrophils and monocytes) at the site of infection or damage following the activation of Pattern Recognition Receptors (PRRs) like Toll like receptors which is activated by cytokine production in a series of inflammatory cascade (Silva *et al.*, 2010; Amdekar *et al.*, 2012).

Oral administration of *W. cibaria* II-1-59, *W.*

*confusa* JMC 1093 and *P. pentosaceus* DSM20336 significantly decrease the paw thickness of the rats (Group C, Group D and Group E). This showed that treatment with these LAB had very consistent anti-inflammatory effect and thus compared favorably with diclofenac sodium (standard NSAID drug). Prostaglandins play significant role in different phases of inflammatory response. Prostaglandins are the majority of molecules responsible for pain and inflammatory diseases. Also, cyclooxygenase (COX) which is involved in prostaglandins synthesis is the rate limiting enzyme. Prostaglandins have not only play pivotal role in inflammation, but these diverse molecules also regulate some critical physiological responses (Dubois *et al.*, 1998). Various studies on immunomodulatory and anti-inflammatory activities of *Weissella* sp. and *Pediococcus* sp. have been reported supporting our findings (Kang *et al.*, 2011, Ahn *et al.*, 2013, Lv *et al.*, 2014, Mizoguchi *et al.*, 2017, Yu *et al.*, 2019, Bian *et al.*, 2020, Yin *et al.*, 2020).

Pro-inflammatory biomarker (CRP) and anti-inflammatory cytokines (IL-10 and TGF- $\beta$ ) were also estimated in this study. CRP is one of the many acute phase reactants that is elaborated in response to inflammation and/or tissue injury. CRP is produced in the hepatocytes of liver in response to IL-6 which is stimulated, in turn, by tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-1 (Ahmed *et al.*, 2012). Studies have shown that certain probiotic LAB have ability to down-regulate pro-inflammatory cytokines (IL-6 and TNF- $\alpha$ ) while they upregulate the production of anti-inflammatory cytokines (IL-10 and TGF- $\beta$ ) (Amdekar *et al.*, 2012). IL-10 is a cytokine with potent anti-inflammatory activity. IL-10 has been shown to be a potent macrophage deactivator, blocking the induced synthesis of TNF- $\alpha$ , IL-1, IL-6, IL-8, and GM-CSF by human monocytes (Trushin *et al.*, 2003). Thus, alleviation of pro-inflammatory cytokines is important for improving inflammatory disorders. Also, previous studies have reported that IL-10 has inhibitory effect on COX-2 synthesis and, hence, the further action of prostaglandin which is a potent inflammatory mediator (Min *et al.*, 2008). Treatment with *W. cibaria* II-1-59; *W. confusa* JMC 1093 and *P. pentosaceus* DSM20336 significantly decreased the secretion of pro-inflammatory

biomarker (CRP) and increased the serum IL-10 and TGF- $\beta$  concentration of Group C, Group D and Group E rats. The present findings are consistent with the results of some other workers. For instance, Yu *et al.* (2019) reported that treatment with *Weissella cibaria* JW15 suppressed the expression of pro-inflammatory cytokines, IL-1, IL-6, and tumor necrosis factor- $\alpha$  in LPS-induced RAW 264.7 cells through regulation of NF- $\kappa$ B activation. Additionally, *W. cibaria* inhibited production of IL-6 and IL-8 in *Fusobacterium nucleatum*-induced KB cells (Kang *et al.*, 2011). The probable mechanism used by *W. cibaria* II-1-59, *W. confusa* JMC 1093 and *P. pentosaceus* DSM20336 could be prostaglandins inhibition due to high production of anti-inflammatory cytokines (IL-10 and TGF- $\beta$ ) after treatment with these LAB. Prostaglandins plays significant role in inflammatory responses. This suggests that these lactic acid bacteria are promising anti-inflammatory agents in the treatment of inflammation.

## CONCLUSION

The results of this present study showed that *W. cibaria* II-1-59, *W. confusa* JMC 1093 and *P. pentosaceus* DSM20336 possess anti-inflammatory potentials and suppresses the effect of inflammation in formalin-induced rat paw oedema, which further ascertain the health beneficial effect of the consumption of 'iru'.

## Competing interest

Authors declare no competing interest.

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