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Original article

Evaluation of isolated probiotics on the efficacy of immune system in male and female Wistar rats

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ABSTRACT

Probiotics were isolated from fruits and vegetables. Microscopic, biochemical, and molecular tests were carried out for the characterization of strains of probiotics. To assess the effects of isolated probiotics on immunity, male and female (15 + 15) Wistar rats **(n = 3)** were randomly distributed into 5 groups: 0-day, negative control, positive control (commercially available Lactobacillus acidophilus-14), laboratory isolated probiotics with accession numbers; Lactobacillus plantarum (MZ707748) and Lactobacillus plantarum (MZ729681), respectively. After hematological investigations, the amounts of IgA and IgG in male and female groups were significantly different ($p < 0.05$). At the same time, the values of Alanine-transaminase (ALT) and Aspartate-aminotransferase (AST) in both genders were average, and there were no differences ($p > 0.05$). Male probiotic-treated groups had decreased levels of interleukin-6, bilirubin, and creatinine, but female probiotic-treated groups had a slight rise in bilirubin and creatinine values ($p = 0.05$). Cellular blood count levels of Hematocrit (HCT) and white blood cells (WBC) in male groups showed considerable differences ($p < 0.05$), while there were no differences ($p > 0.05$) in female groups. Levels of Red blood cells (RBC) and mean corpuscular hemoglobin concentration (MCHC) showed distinct changes ($p < 0.05$) in female groups, while these values were insignificant changes ($p > 0.05$) among male groups. There were considerable differences between the control and groups that were given probiotics. Histopathological results showed no damage to the liver and thymus. A fecal examination of rats was used to examine the viability and survival of Lactobacilli. Based on blood tests, it was observed that the immune system was boosted and improved in probiotic-treated groups compared to control groups.

1. Introduction

Probiotics are "living microbes that, when taken in sufficient proportions, confer a health assistance to the host" by promoting the development of other microbes, altering mucosal and total immunity, and attempting to improve the gut system's dietary and bacterial balance. While bacteria, fungi, and yeast can all be used as probiotics, bacterial strains, primarily those that produce lactic acid, are the most popular (Shokryazdan et al., 2021). Fruits and veggies are considered healthy foods and make an excellent vehicle for functional foods because they are packed with vitamins, minerals, phytochemicals, antioxidants, dietary fibers, and other helpful substances (Aspri et al., 2020). Fruits are a suitable substrate for the development of probiotic bacteria. Still, because of the acidic conditions of the fruit, which the probiotic bacteria need to be safe from, it is more difficult for these microbes to live in such a configuration than it is in dairy products (Pereira et al., 2018). Lactic acid bacteria (LAB) are one of the unique probiotics and have been stated to be a valuable adjunct to food, eventually encouraging health and strength to a massive extent

Lactobacilli and *Bifidobacteria* have been shown to reduce *Helicobacter pylori,* Escherichia *coli, Listeria monocytogenes, Salmonella,* and *Rotavirus* (Bermudez-Brito et al., 2012). As a result, probiotics activate particular antibodies against rotavirus in children, potentially lowering the prevalence of diarrhea (Habil 2015). Probiotics have been shown to create a variety of bacteriocins, including niacin (de Arauz et al., 2009), which are the main antibacterial mech-

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anisms. Acidophillin, lactocidin, and acidolin are produced by *Lactobacillus acidophilus*. At the same time, lactolin is derived from *L. plantarum* (Vila et al., 2010), and these microbial metabolites can affect inflammation and autoimmunity (Yousefi et al., 2019).

The protective influence of immunobiotic Lactobacilli has been well documented; indicating that they can help the host enhance some immune processes (Hevia et al., 2015). Adaptive immunity is linked to the immunoglobulins produced in response to antigen exposure. B cells predominately produce them. Additionally, these immunoglobulins are broken down into IgE, IgG, and IgA groups. The primary target for defense is IgAs. They can be found in perspiration, mucosal secretions, breast milk, saliva, and tears.

Additionally, they support the control of commensal bacteria at mucosal locations, and when an infection occurs, they defend the body (Hachimura et al., 2018). T-cells are another class of specialized or adaptive immune cells. They were born in the large and small intestines. Alpha and beta strands are present in the receptors. These are further categorized for CD4 and CD8 cells according to the expression levels. T helper cells (Th) are CD4 + T cells that support immunological processes by producing cytokines like IL-4, IL-17, and IFN (interferon) (Ashaolu, 2020). The most significant part of innate immunity is played by Natural Killer (NK) Cells, which are Type-1 Innate Lymphocytes (ILCs) and can be lethal to immune cells (Ardain et al., 2020).

According to Kim et al. (2020a, 2020b), *Lactobacillus* strains can boost the release of IL-12, which triggers the NK cell activation and IFN secretion, resulting in an innate immune reaction. Probiotics may play a variety of functions, one of which is to modify immune responses in various diseases. By producing immunomodulatory and inhibitory cytokines, tellurogenic probiotics can contribute significantly to preserving tolerance in autoimmune and inflammation-related diseases (Zhai et al., 2021). Neurogenic probiotics have been shown to have an impact on immune-mediated lupus erythematous in studies. To ameliorate symptoms and generate tolerant DCs and Treg in lupus mice using *L. rhamnosus* and *L. delbrueckii* (Zeng et al., 2019), the positive immune modulation impacts changed the immune response specificity i.e. tolerance to allergens, rheumatoid arthritis (Burmester et al., 2017), and inflammatory bowel disease (IBD) (Caruso et al., 2020). According to findings, multiple sclerosis is one of the most harmful autoimmune illnesses connected to instability in the microbiota in the gut.

The influence of dietary probiotics on the gastrointestinal tract is primarily due to the fecal survival of the consumed strains. They populate the stomach for a short time before departing once the food has been swallowed. Antibacterial compounds with broad antimicrobial action, such as reuterin or plantaricins, are passed through probiotics, or the bacterial gut composition remains more consistent during probiotic treatment, and this has been linked to improved disease symptoms (Ceapa et al., 2013).

The protection of probiotic strains, like *L. plantarum*, is typically presumed based on previous experiences consuming significant amounts of fermented foods, devoid of experiments conducted, according to Siciliano et al. (2021). The purpose of this study is to evaluate more about the effects of orally administered L. plantarum on rat hematological and serum parameters. Furthermore, the effective doses that provide a health-promoting effect in rats are investigated.

2. Materials and methods

2.1. Sample collection and bacterial isolation

Sampling was done from different areas of Lahore (Table S1). These samples were washed with tap water, followed by a rinse with saline (0.9%) to avoid any contamination. A small section of the fruit samples under investigation was manually chopped with a sterile cutter. The citrus fruits (orange and lemon) were squeezed, and 1 mL of this fruit extract was inoculated into 9 mL De-Man, Rogosa, and Sharpe (MRS) broth and incubated at 37 °C for 24 h. Suitable sample dilutions were prepared, and only 50 µL was spread on MRS agar plates. Every plate was incubated for 48 h till growth. Bacterial isolates were picked by using sterilized loops and streaked on plates. Then each plate was placed at 37 °C in an incubator for 48 h (Panghal et al., 2018; Li et al., 2020).

2.2. Microscopic examination

Gram's staining aimed to differentiate between gram-negative and gram-positive bacteria. Gram staining was used to identify the shape of bacteria like rod-shaped bacteria are bacilli. Endospore staining was used to determine the spore formation of bacteria. Gram's and endospore staining was done by the protocol of (Benson 2002; Dabiré et al., 2022).

2.3. Biochemical characterization

The catalase test was carried out to determine whether probiotic microorganisms can degrade hydrogen peroxide (Jena et al., 2013). To assess the motility of strains, a motility test was performed. A casein hydrolysis test was performed by growing LAB on skim milk agar plates and checking plates for casein hydrolysis. A glucose fermentation test is to verify the micro-organisms' capacity to ferment carbohydrates through gas and acid. The antibiotic susceptibility of strains was evaluated through disc diffusion (Haghshenas et al., 2014; Dabiré, et al., 2022).

2.4. Molecular characterization

2.4.1. Isolation of genomic DNA

DNA of bacterial strains was isolated using the CTAB method followed by the protocol of (William et al., 2012). Isolated DNA was re-suspended in 50 µL TE buffer and confirmed by agarose gel (1%) electrophoresis. Now DNA was stored at − 20 °C.

2.4.2. Primers used

Following primers were used for 16S rRNA characterization.
8F (Forward primer): 5′ AGAGTTTGATCCTGGCTCAG 3′.
1492R (Reverse primer): 5′ GGTTACCTTGTTACGACTT 3′.

2.4.3. PCR- based amplification of 16S rRNA gene

The PCR-optimized conditions for amplifying PCR products were denaturation at 94 °C for 4 min, annealing temperature of 55 °C for 30 sec, extension at 72 °C for 35 sec, and final extension for 5 min with 35 cycles.

2.4.4. DNA purification from the reaction mixture

DNA purification was done following the protocol by Smith et al. (1995) through a silica bead DNA gel extraction kit (K0513). After the gene was cleaned, the PCR product was sent to the 16S rRNA Gene Sequencing Centre (ABI), Malaysia.

2.5. Experimental animal model

Female and male (15 + 15) (n = 3) Wistar albino rats (Rattus norvegicus), 5–6 weeks old (90–200 g), were bought from the University of Veterinary and Animal Sciences (UVAS), Lahore. Rats were habituated for 10 days with 30–40 percent humidity in an animal house (GCU) in Lahore, Pakistan. Rats were given conventional chaw with autoclaved distilled water. Daily 0.4 mL gavage was used to administer probiotics to rats for 30 days (Lollo et al., 2013). Group C was fed on L. acidophilus-14, and treated groups (D, E) were provided on L. plantarum MZ707748 and L. plantarum

MZ729681, respectively. Colony forming unit (CFU) of groups C, D, and E were taken into account using the pour plate protocol, which relates to the dilution series of the basic stock, Lactobacillus acidophilus (1.25×10^{12} /mL), L plantarum MZ707748 (8.25×10^{12}/mL) and L. plantarum MZ729681 (5.00×10^{12}/ml) groups. The groups designated were A (0 days), B (Negative control), C (Positive control), D (L. plantarum MZ707748), and E (L. plantarum MZ729681). The commercial probiotic strain L. acidophillus-14 was used as a positive control (Fig. 1).

2.6. Blood sampling

Rats were sacrificed for sampling after one month of probiotic dose. Blood was taken through a cardiac puncture procedure and divided into two tubes containing an ethylenediaminetetraacetic acid (EDTA) coating for hematological analysis and a plain tube for serum analysis. White blood cells (WBC), red blood cells (RBC), platelets (PLT), hemoglobin concentration (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), lymphocytes, basophils are the entire hematological parameters. In blood serum levels, subsequent factors were evaluated: Globulin, albumin, total protein, interleukin-6, IgA, IgG, alanine-transaminase (ALT), bilirubin, creatinine and aspartate-aminotransferase (AST). The automated Huma Count 30TS/ Hu123Wma Count 80TS analyzer (Cat No. 16420/801 Human, Germany) was employed to perform the Whole Blood Count test on all samples. The serum chemistry was analyzed on equipment (PDL- PAT –EQP-005- Is 2014, Italy).

2.7. Histopathology

Rats were dissected, and respective organs (thymus and liver) were kept in 10% formalin. The organs were dehydrated by repeatedly immersing them in a rated combination of ethanol and water (70–100%). A solvent combined with the fixing medium was used to substitute the ethanol. When xylene was used to enter the tissues, they became transparent (clearing). The xylene-infused tissue was put in melted paraffin and kept at 58–60 °C in the oven (embedding). The solvent evaporated thanks to the heat, and paraffin occupied the gaps inside the tissues. After being taken out of the kiln, the tissue's impregnating paraffin solidified. After being suspended on water and moved to a glass slide, the portions (5 m) were stained with hematoxylin and eosin stains. Under a microscopic examination with a magnification of 10X, the plates were examined (Garuba et al., 2023).

2.8. Fecal analysis of probiotic-treated rats

Fecal samples were collected on 0 days and at the end of the trial on the 30th day to confirm the probiotic consumption. Feces were dissolved in a sterile tube containing 10 mL of PBS (Phosphate buffer saline). The samples were centrifuged at 10,000 rpm for 10 min. The incubated supernatant was then spread on MRS agar plates. On the MRS agar dish, the growth became visible. Colonies were injected into MRS broth and were tolerable to grow for 24 h at 37 °C. Additional research was done to prove probiotic species (Inoue and Ushida 2003).

2.8.1. Fecal biochemical tests

The antibacterial activity of isolated fecal strains was evaluated through pathogenic microbes (Haghshenas et al., 2014; Jafarpour et al., 2015).

2.9. Statistical analysis

One-way analysis of variance (Mean ± S.D) was used to analyze the data. Multiple comparisons were made using Tukey's test. For all statistical studies, the SPSS program version 20.0 was utilized (Statistical Pack initial Age for Social Sciences). $P < 0.05$ was used as the significance level, and the result was given as mean and standard deviation. GraphPad 7.0 was used only to make graphs (Prism).

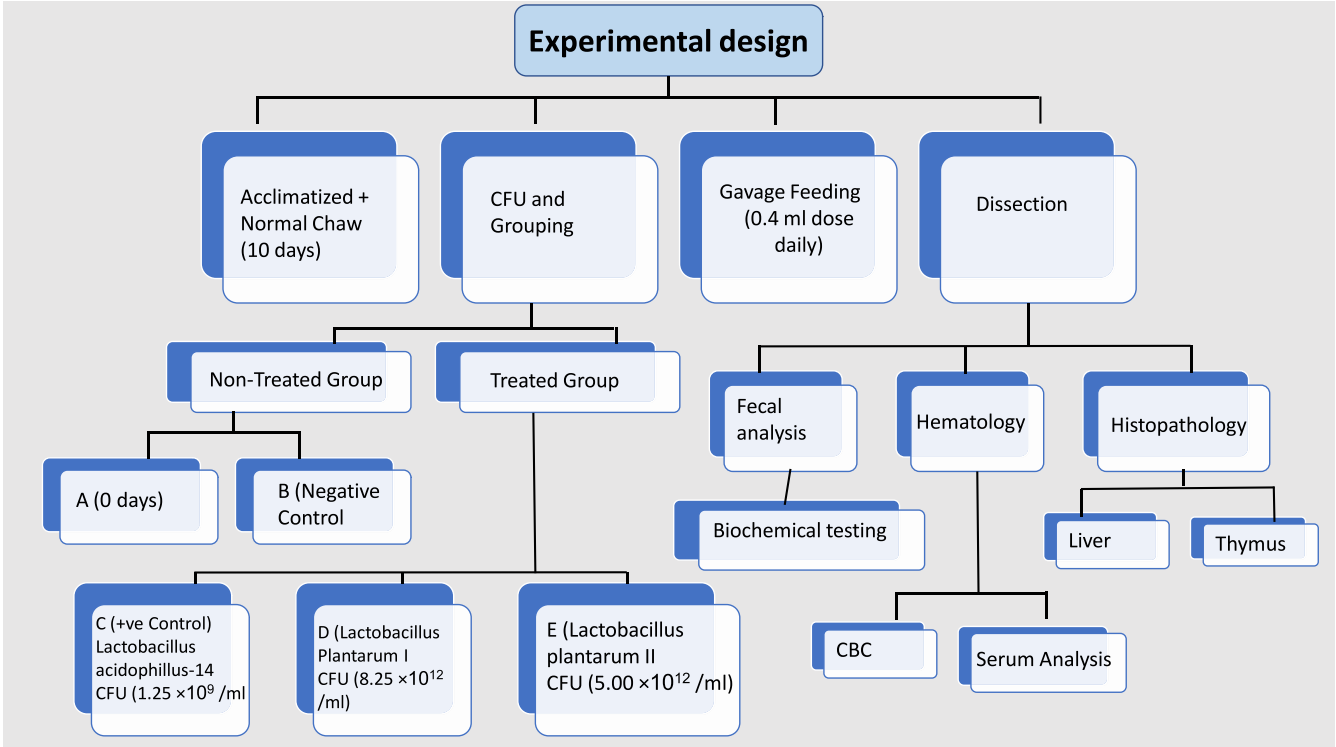

Fig. 1. Experimental design of the current research work.

Table 1
Effect of probiotic strains on average weight of Wistar rats.

Groups	Gender	Initial body Weight (g)	Final body weight (g)	Weight gain (g)
A	M	91.7 ± 5.1^{c}	91.7 ± 5.1^{d}	0.0 ± 0.0^{c}
	F	83.1 ± 6.8^{a}	83.1 ± 6.8^{e}	0.0 ± 0.0^{e}
B	M	154 ± 7.6^{ab}	248.6 ± 12.8^{bc}	99.6 ± 7.5^{bc}
	F	127 ± 10^{a}	200 ± 10^{bcd}	73.0 ± 10^{abc}
C	M	166.3 ± 5.5^{c}	243.6 ± 7.7^{c}	77.3 ± 5.7^{ab}
	F	125.3 ± 7.5^{a}	214.0 ± 20.4 ^{cd}	88.6 ± 19.3 ^{cd}
D	M	158.3 ± 5.7^{ab}	216.6 ± 10.2^{abc}	58.3 ± 8^{ab}
	F	111.6 ± 10.5^{a}	227.0 ± 20.5^{a}	115.3 ± 9.7^{ab}
E	M	159.6 ± 17.3^{ab}	234.3 ± 2.3^{abc}	74.6 ± 15^{ab}
	F	98.6 ± 7.5^{a}	225.0 ± 10.4^{ab}	126.3 ± 3.21^{a}
p value	M	0.00	0.00	0.01
	F	0.007	0.00	0.00

*Data represented the (Mean ± S.D) weight of male and female rats.

Table 2
Effect of probiotic strains on relative weight of organs.

Groups	Gender	Liver (g)	Thymus (g)
A	M	2.65 ± 0.1^{bc}	0.16 ± 0.0^{a}
	F	3.1 ± 0.01^{a}	0.12 ± 0.01^{a}
B	M	5.70 ± 1.1^{a}	0.29 ± 0.1^{ab}
	F	7.0 ± 1.2^{a}	0.32 ± 0.1^{a}
C	M	6.37 ± 0.9^{bc}	0.32 ± 0.06^{b}
	F	6.06 ± 0.6^{a}	0.32 ± 0.09^{a}
D	M	8.97 ± 0.3^{ab}	0.29 ± 0.01^{ab}
	F	6.53 ± 0.6^{a}	0.44 ± 0.03^{a}
E	M	5.57 ± 0.5^{c}	0.26 ± 0.1^{ab}
	F	6.25 ± 0.9^{a}	0.37 ± 0.05^{a}
p value	M	0.004	0.219
	F	0.000	0.002

*Data is presented as (mean ± S.D) of male and female Wistar rats' organ.

3. Results

3.1. Bacterial isolation

Two probiotics were isolated from 5 samples, named GCU-DAB-Z-1, GCU-DAB-Z-2, GCU-DAB-Z-3, GCU-DAB-Z-4, and GCU-DAB-Z-5 (Table S1; Fig. S1).

3.2. Morphological characterization

Two probiotic strains were isolated, GCU-DAB-Z-3 and GCU-DAB-Z-4, that formed round, creamy white, smooth, medium, and large colonies on the MRS agar plate. Gram staining showed that cultures of bacterial isolates were stained purple, and the rod formed. This indicated that all cultures of isolated strains were gram-positive bacillus. In the case of endospore staining, it was noticed that all the bacterial isolates were without spores (Table S2).

3.3. Biochemical characterization

The catalase test performed the biochemical characterization of two isolates, which all showed negative results. The catalase test results showed that all bacterial isolates could not degrade hydrogen peroxide (H_2O_2), meaning they are catalase negative. The results of the motility test showed that all the isolates were non-motile. After performing a casein hydrolysis test, it was observed that all the bacterial isolates showed negative results, as no clear zone was formed around the streak. A glucose fermentation test

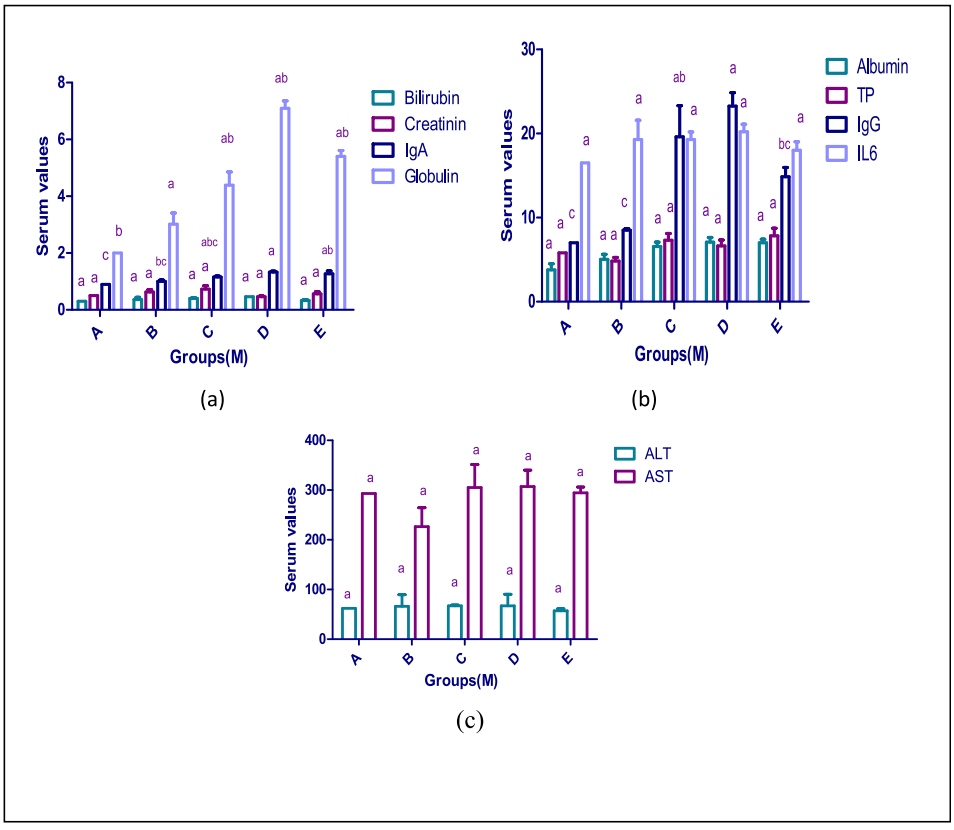

Fig. 2. Serology of male Wistar rats between groups (A, B, C, D & E), (a) Globulin, IgA, Creatinin and Bilirubin values (b) Albumin, Total protein (TP), IgG and Interleukin-6 (IL-6) levels (c) Alanine-transaminase (ALT) and Aspartate transaminase (AST) values. ^{abc}Alphabet on bar shows difference between groups ($p<0.05$).

was performed, and the results of the fermentation test also showed that all isolates were positive for various carbohydrate fermentation tests (Table S2).

3.4. Antibiotic susceptibility

It was seen that strain GCU-DAB-Z-4 was resistant towards amoxicillin (AX-25), penicillin (P-10), erythromycin (E-15), gentamicin (CN-120), ciprofloxacin (CIP-5), and cefixime (CFM-5) (Fig. S2). GCU-DAB-Z-3 was mildly susceptible to gentamicin. A new bacterial culture was spread along with six different antibiotic discs on each Petri plate to determine antibiotic resistance. Sensitive to antibiotics strains show > 21 mm, mildly susceptible with a diameter of 16–20 mm, and resistant strains with < 15 mm radius (Table S3).

3.5. Molecular characterization of bacterial isolates

3.5.1. Isolation of genomic DNA

For gel electrophoresis, 3 μL of genomic DNA was loaded, and the gel electrophoresis showed the sharp bands of GCU-DAB-Z-3 and GCU-DAB-Z-4 (Fig. S3a).

3.5.2. Amplification of 16S rRNA gene

Using forward and reverse primers, conserved regions of the 16S rRNA gene were enlarged with the help of genomic DNA in the isolated bacterial strains. PCR product of each isolate was then envisioned on agarose gel with a DNA ladder (Fig. S3b).

3.5.3. Sequence analysis

The nucleotide sequence of the total length 16S rRNA gene was done to identify *L. plantarum* isolates up to the specie level. The dendrogram of the sequences mentioned above shows that GCU-DAB-Z-3 and GCU-DAB-Z-4 have 98% homology to other *L. plantarum* strains and DAB 234 strains, respectively (Fig. S4a,b).

3.6. Body weight changes

On the first and last days of the trial, the weights of every rat were recorded. None of the rats displayed any remarkable physical or behavioral modifications or unexpected deaths throughout the trial. Table 1 shows an initial, final, and weight gain in both genders, but there was a significant increase in body weight gain in the females as compared to the males. While treated groups *L. plantarun* MZ707748, *L. plantarun* MZ729681, and *L. acidophilus*-14 raise these parameters but are insignificant compared with the control. The results of the final rat weight were significant ($p = 0.00$). *L. plantarum* has demonstrated its effectiveness as a probiotic with anti-obesity impacts, at least in animal research.

3.6.1. Changes in relative weight organs

The findings from Table 2 demonstrate no significant differences between the three strains in the proportional weight of the liver and thymus. This indicated that *L plantarum* strains and *L acidophilus* did not cause any adverse effects on organs. *L. plantarum* MZ707748 had the highest globulin value compared to other groups, whereas IgA was frequently similar among groups. The

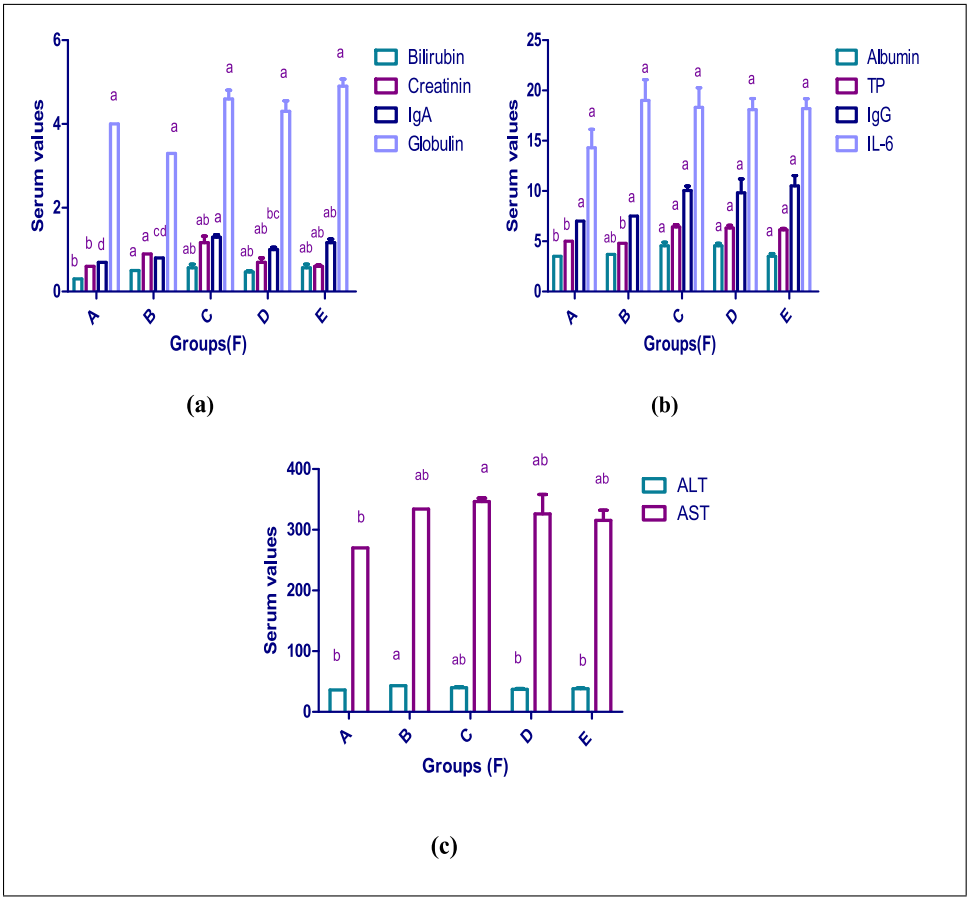

Fig. 3. Serology of female Wistar rats between groups (A, B, C, D & E), (a) Globulin, IgA, Creatinine and Bilirubin values (b) Albumin, total protein (TP), IgG and Interleukin-6 (IL-6) levels (c) Alanine transaminase (ALT) and Aspartate transaminase (AST) values. [abc]Alphabet on bar shows the significance difference between groups ($p<0.05$).

laboratory-isolated microbes groups (D, E) had lowered creatinine and bilirubin concentrations shown in Fig. 2(a) while albumin, TP, and IL-6 had no change among groups. In contrast, IgG concentration slightly increased in *L. plantarum* MZ707748 shown in graph (Fig. 2b). ALT and AST concentrations were not different in any group (Fig. 2c).

L. plantarum MZ729681 had the highest value of globulin compared to other groups. At the same time, IgA levels increased between treated groups. The laboratory-isolated microbes groups (D, E) had lowered creatinine and bilirubin concentration (Fig. 3a). Albumin, TP, and IgG concentrations were slightly increased among groups. At the same time, IL-6 level was slightly decreased between treated groups (Fig. 3b). ALT and AST concentrations were not different in the group (Fig. 3c).

Hb had the highest concentration in group D. Increased hemoglobin levels was determined in probiotic groups. The highest neutrophil values were seen in group E. It had nearly similar effects in groups C and D. In contrast; lower values were observed in groups A and B. Group D had the highest values of HCT. In contrast, its lowest values were observed in group E. MCH concentrations were similar in all groups except group A (Fig. 4a). Group D had the highest values of red blood cells. Eosinophil concentrations were highest in group E, and basophils had similar concentrations in all groups (Fig. 4b). Platelets had the highest concentration in group E. MCHC had similar concentrations in all groups. While lymphocytes and MCV levels were seen to be significantly different among all groups (Fig. 4b).

The highest concentrations of Hb levels were seen in probiotic groups. The lowest concentrations of neutrophils were observed in females compared to male Wistar rats. Group C had the highest value of MCH as compared to D and E. MCH concentration was higher in groups D, E, and F as compared to A and B in Fig. 5a. In comparison to the other groups, group C had higher RBC concentrations, while Group D had the highest WBC concentrations. Basophil had higher concentrations in group B than in treated groups. Eosinophil had similar concentrations in all groups (Fig. 5b). MCHC, MCV, and lymphocytes had observed similar concentrations in all groups. In comparison, groups D and E exhibited the highest levels of platelets compared to groups A and B shown in (Fig. 5c).

3.6.2. Serum chemistry analysis

Between the groups of male Wistar rats, the serum parameters were Globulin, IgA, IgG, and total protein statistically significant ($p<0.05$). At the same time, while albumin, ALT, AST, bilirubin, creatinine, and interleukin-6 showed insignificant change ($p>0.05$). In the case of female rats, albumin, globulin, IgA, IgG, total protein, bilirubin, and creatinine exhibit significant variation ($p<0.05$), but IL-6, AST, and ALT appeared to have no difference ($p>0.05$) (Table 3). All of the measurements were within normal physiological limits.

3.6.3. Complete blood count parameters

Among the groups of male Wistar rats, values of Hb, WBC, HCT, MCH, MCV, neutrophils, and eosinophils, showed significant differ-

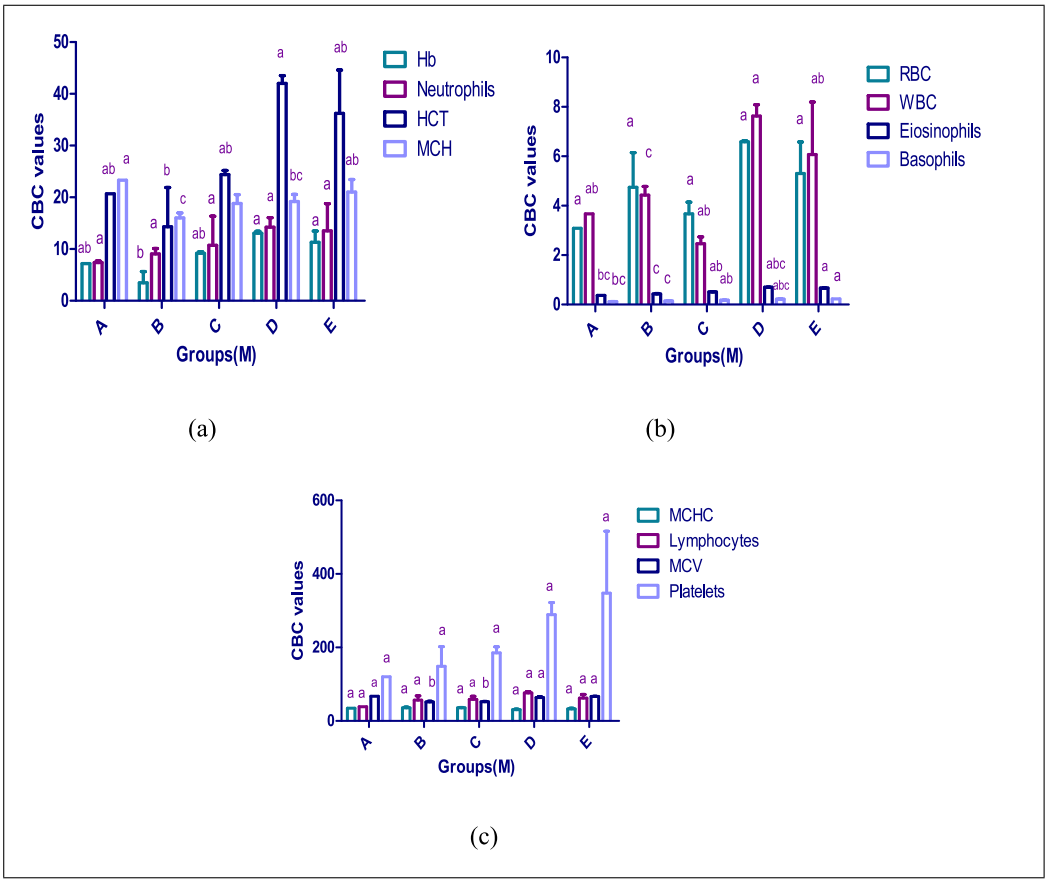

Fig. 4. Hematological findings of male Wistar rats between groups (A, B, C, D & E) (a) hemoglobin concentration (Hb), neutrophils, hematocrit (HCT) and mean corpuscular hemoglobin (MCH) (b) red blood cell (RBC), white blood cell (WBC), basophils and eiosinophils (c) mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), lymphocytes and Platelets. [abc]Alphabet on bar shows the significance difference between groups ($p<0.05$).

ence ($p<0.05$), and RBC, MCHC, basophils, lymphocytes, and Platelets revealed no significant differences ($p>0.05$). While the groups of female Wistar rats, values of Hb, RBC, MCHC, MCH, MCV, and lymphocytes showed significant differences ($p<0.05$), but HCT, WBC, platelets, and neutrophils were insignificant values ($p>0.05$) (Table 4).

3.7. Fecal antimicrobial activity results

Probiotics showed maximum resistance for *Pseudomonas aeruginosa,* while their effects against *E. coli* and *S. aureus* were also observed in (Table S3). The isolates from probiotics-treated groups (*L. plantarum* MZ707748, *L. plantarum* MZ729681, and *L acidophilus*-14) showed significant resistance towards the pathogenic strains *P. aeruginosa, Staphylococcus aureus,* and *E. coli.* The inhibition zones were measured, while the untreated group displayed the least amount of action and pathogen survival. Clear zones were observed on all the plates. All the experienced isolates could co-aggregate with pathogens, but groups (A, B) were less effective on the side of pathogens (Fig. S5).

3.8. Histopathological analysis

The number of hepatocytes was found to be higher in groups that were given probiotics. Hepatocytes are almost normal, and no tissue changes were seen. Hepatic cords are normal and have a polygonal shape. No changes in the cytoplasm and nuclei are seen. Sinusoids and portal area are also normal. But in the control group, hepatocytes are shrunken, and sinusoidal space is vast (Fig. 6).

No fibrosis was seen in the treated groups, and the number of Th1 and Th2 cells in the thymus rose. Cells are normal in morphology and more in number. Nuclear and cytoplasmic morphology is intact. No architectural changes are seen. The morphology of glands is also intact between treated groups. No histological liver or thymus damage was observed in any groups. Cells are regular in shape, and morphology and architecture are almost normal. No tissue changes were seen in the control groups. On the compound microscope, the results of the histopathological investigation of the liver and thymus were visible at a magnification of 10X (Fig. 7).

4. Discussion

Probiotic effectiveness and possible health benefits have been demonstrated in animal models, in vitro experiments, and human clinical research (James and Wang, 2019). The manufacture of probiotic food is carried out by probiotic bacteria, which are crucial for the development of gut flora. It also benefits the digestive system by boosting a person's resistance to microorganisms that are harmful to human health. These bacteria can be bred to produce a variety of pharmaceuticals in the development of new functional foods (Shahriar et al., 2019). The effect of probiotics on pathogenic

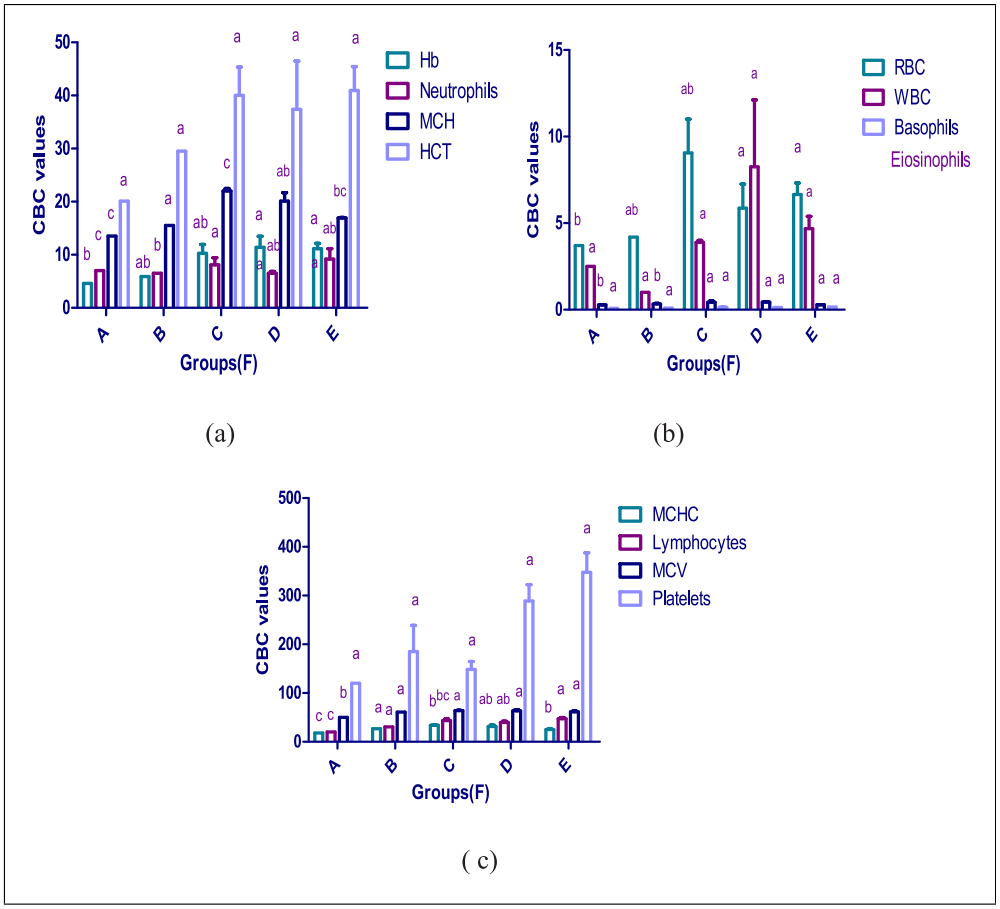

Fig. 5. Hematological values of average weight of Female Wistar rats between groups (A, B, C, D & E) (a) red blood cell (RBC), Eiosinophils, white blood cell (WBC) and basophils (b) hemoglobin concentration (Hb), Hematocrit (HCT), Neutrophils and mean corpuscular hemoglobin (MCH) (c) Lymphocytes, Platelets, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC). [abc]Alphabet on bar shows the significance variation among groups ($p<0.05$).

microorganisms is related to several mechanisms, including antimicrobial secretion, competitive adhesion to epithelium and mucosa, reinforcement of intestinal epithelial barrier, and immune system regulatory impact (Javanshir et al., 2021). The present study isolated and characterized local probiotics from different vegetables and fruits. Various researchers have reported the isolation of probiotics from different ecological environments (Dabiré, et al., 2022; Roza et al., 2022).

In the current investigation, rats provided with L. plantarum, a probiotic microbe, showed improved health based on their hematological state. These findings indicate that probiotics have immuno-stimulatory effects. In another study, the serum biochemistry findings revealed that all groups had hematological parameters within normal limits (Kim et al., 2020). Sakai et al. (2021) described that white blood cells (WBCs) such as basophils, monocytes, eosinophils, and lymphocytes neutrophils regulate immune systems and protect the body from allergens, infections, and disease. The same findings are reported in the present study.

Lactobacillus sp. Lp6 adherence to rat mucus is regulated by mannose-specific hold proteins, which are bundled to cell surface elements and are critical for competing with bacterial binding sites in the gut, preventing pathogen colonization (Sun et al., 2007). Jafarpour et al. (2015) reported that two bacterial strains (L. plantarum and B. coagulans) along with prebiotic inulin play a protec-

Table 3
Serum chemistry test of male and female Wistar rats between groups.

Parameters	Gender	A	B	C	D	E	p Value
Albumin	**M**	4.5 ± 1.21^{a}	5.03 ± 1.18^{a}	6.03 ± 0.92^{a}	6.26 ± 0.92^{a}	4.83 ± 1.10^{a}	0.263
(g/dL)	**F**	3.5 ± 0.0^{b}	3.7 ± 0.0^{ab}	4.56 ± 0.60^{a}	4.56 ± 0.41^{a}	3.50 ± 0.50^{b}	0.011
Globulin	**M**	2 ± 0.0^{b}	3.48 ± 0.69^{a}	3.13 ± 0.47^{ab}	3.40 ± 0.45^{ab}	2.8 ± 0.76^{ab}	0.045
(g/dL)	**F**	4.0 ± 0.0^{a}	3.30 ± 0.0^{a}	4.60 ± 0.36^{a}	4.30 ± 0.43^{a}	4.90 ± 0.30^{a}	0
IgA	**M**	0.90 ± 0.0^{c}	1.00 ± 0.10^{bc}	1.16 ± 0.05^{abc}	1.33 ± 0.05^{a}	1.26 ± 0.20^{ab}	0.003
(ug/ml)	**F**	0.70 ± 0.0^{d}	0.80 ± 0.0 ^{cd}	1.30 ± 0.10^{a}	1.0 ± 0.10^{bc}	1.16 ± 0.15^{ab}	0
ALT	**M**	62.00 ± 0.0^{a}	66.0 ± 40.9^{a}	67.00 ± 3.0^{a}	67.33 ± 40.0^{a}	57.0 ± 7.0^{a}	0.985
(uL)	**F**	36.0 ± 0.0^{b}	43.3 ± 0.0 ^{a}	39.6 ± 2.51^{ab}	37.3 ± 2.3^{b}	38.6 ± 1.7^{b}	0.065
AST	**M**	293.0 ± 0.0^{a}	266.6 ± 65.4^{a}	305.6 ± 80.5^{a}	307.0 ± 33.1^{a}	294.6 ± 20.4^{a}	0.851
(uL)	**F**	270 ± 0.0^{b}	334.0 ± 0.0^{ab}	346.3 ± 10^{a}	330.0 ± 59.4^{ab}	315.3 ± 29.1^{ab}	0.076
Billirubin	**M**	0.3 ± 0.0^{a}	0.36 ± 0.15^{a}	0.46 ± 0.05^{a}	0.40 ± 0.00 ^{a}	0.33 ± 0.05^{a}	0.162
(mg/dL)	**F**	0.30 ± 0.0^{b}	0.50 ± 0.00 ^{a}	0.56 ± 0.15^{ab}	0.46 ± 0.05^{ab}	0.57 ± 0.15^{ab}	0.046
Creatanine	**M**	0.50 ± 0.0^{a}	0.63 ± 0.15^{a}	0.73 ± 0.20^{a}	0.46 ± 0.05^{a}	0.56 ± 0.15^{a}	0.198
(mg/dL)	**F**	0.60 ± 0.0^{b}	0.90 ± 0.0^{a}	1.10 ± 0.20^{ab}	0.70 ± 0.17^{ab}	0.63 ± 0.05^{b}	0.002
IgG	**M**	7 ± 0.0^{c}	8.5 ± 0.50^{c}	19.6 ± 6.52^{ab}	23.26 ± 2.83^{a}	14.0 ± 2.0^{bc}	0.001
(g/L)	**F**	7 ± 0.0^{a}	7.5 ± 0.00^{a}	10.06 ± 0.73^{a}	9.83 ± 2.36^{a}	10.50 ± 1.80^{a}	0.031
IL-6	**M**	16.5 ± 0.00^{a}	19.3 ± 2.7^{a}	19.3 ± 2.5^{a}	20.2 ± 4.2^{a}	18.0 ± 1.5^{a}	0.31
(pg/ml)	**F**	14.3 ± 1.83^{a}	19.0 ± 2.15 ^{a}	18.3 ± 3.3^{a}	18.1 ± 3.6^{a}	18.2 ± 4.1^{a}	0.33
TP	**M**	5.8 ± 0.0^{a}	4.83 ± 0.76^{a}	7.3 ± 1.5^{a}	6.63 ± 1.26^{a}	7.83 ± 1.60^{a}	0.031
(g/dL)	**F**	5.0 ± 0.00^{b}	4.8 ± 0.00^{b}	6.4 ± 0.40^{a}	6.3 ± 0.47^{a}	6.13 ± 0.25^{a}	0

A- 0 day, B- Negative control, C- Positive control, D- *L. plantarum* MZ707748, E- *L. plantarum* MZ729681.
All values are represented as the (Mean ± S.D) of Wistar rats, M: male; F: female.
^{a,b,c} $P < 0.05$ compared with the control group by ANOVA followed by Tuckey's test.

Table 4
Complete blood count of male and female Wistar rats between groups.

Parameters	Gender	A	B	C	D	E	p Value
Hb	**M**	7.2 ± 0.0^{ab}	3.4 ± 0.55^{b}	9.2 ± 3.7^{ab}	13 ± 0.6^{a}	11.3 ± 3.7^{a}	0.005
(g/dL)	**F**	4.6 ± 0.0^{b}	5.9 ± 0.0^{ab}	10. ± 2.9^{ab}	11 ± 3.4^{a}	11.2 ± 1.6^{a}	0.008
RBC	**M**	3.1 ± 0.0^{a}	3.6 ± 0.8^{a}	4.7 ± 2.4^{a}	6.6 ± 0.07^{a}	5.3 ± 2.2^{a}	0.111
(10^{6}/uL)	**F**	3.7 ± 0.0^{b}	4.2 ± 0.0^{a}	9.1 ± 3.3^{ab}	5.8 ± 2.3^{ab}	6.6 ± 1.1^{ab}	0.042
HCT	**M**	20.7 ± 0.0^{ab}	14 ± 1.5^{b}	24 ± 13.0^{ab}	42 ± 2.6^{a}	36.2 ± 14.5^{ab}	0.018
(%)	**F**	20 ± 0.0^{a}	29 ± 0.0^{a}	40 ± 9.2^{a}	37.4 ± 15.7^{a}	40.9 ± 7.8^{a}	0.076
MCHC	**M**	34.7 ± 0.0^{a}	35.5 ± 1.5^{a}	35.9 ± 4.0^{a}	30.9 ± 1.9^{a}	32.9 ± 2.9^{a}	0.148
(g/dL)	**F**	18 ± 0.0^{c}	26.7 ± 0.0^{a}	33.83 ± 1.7^{b}	32 ± 4.8^{ab}	25.2 ± 2.3^{b}	0
MCH	**M**	23.3 ± 0.0^{a}	16 ± 1.7^{c}	18.8 ± 0.8 ^{bc}	19 ± 1.3^{bc}	21 ± 2.4 ^{ab}	0.002
(pg)	**F**	13.5 ± 0.0^{c}	15.5 ± 0.0^{a}	22 ± 0.8^{c}	20 ± 2.7^{ab}	17 ± 0.15^{bc}	0
WBC	**M**	3.6 ± 0.0^{ab}	2.4 ± 0.4^{b}	4.4 ± 0.5^{ab}	7.6 ± 0.7^{a}	6.1 ± 3.6^{ab}	0.030
(10^{3}/uL)	**F**	2.5 ± 0.0^{a}	1.0 ± 0.0^{a}	3.9 ± 0.2^{a}	8.25 ± 6.7^{a}	4.6 ± 1.25^{a}	0.121
Plateletes	**M**	120 ± 0.0^{a}	148 ± 28.0^{a}	185 ± 92.8^{a}	289 ± 57.4^{a}	347. ± 291.7^{a}	0.293
(10^{3}/uL)	**F**	120 ± 0.0^{a}	102 ± 0.0^{a}	235 ± 45.5^{a}	345 ± 268.4^{a}	423 ± 284.4^{a}	0.19
Lymphoytes	**M**	38 ± 0.0^{a}	56.8 ± 15.1^{a}	58.6 ± 20.1^{a}	76.2 ± 6.29^{a}	62 ± 17.6^{a}	0.089
(%)	**F**	20 ± 0.0^{c}	30.5 ± 0.0^{a}	43 ± 6.2^{bc}	39.6 ± 4.6^{ab}	47.3 ± 4.04^{a}	0
Neutrophils	**M**	7.39 ± 0.5^{a}	9.06 ± 2.1^{a}	10.7 ± 1.8^{a}	14.2 ± 3.2^{a}	13.5 ± 5.7 ^{a}	0
(%)	**F**	5.7 ± 0.4^{a}	7.5 ± 1.05^{a}	9.8 ± 0.61^{a}	8.6 ± 0.32 ^{a}	9.2 ± 0.64 ^{a}	0.007
Eosinophils	**M**	0.3 ± 0.05^{bc}	0.4 ± 0.18^{c}	0.5 ± 0.1^{ab}	0.7 ± 0.10 ^{abc}	0.6 ± 0.3^{a}	0
(%)	**F**	0.28 ± 0.01^{a}	0.33 ± 0.07^{a}	0.43 ± 0.12^{a}	0.45 ± 0.04^{a}	0.29 ± 0.01^{ab}	0.032
Basophils	**M**	0.12 ± 0.4^{bc}	0.15 ± 0.07^{c}	0.18 ± 0.04^{ab}	0.23 ± 0.1 ^{abc}	0.23 ± 0.17^{a}	0.321
(%)	**F**	0.08 ± 0.00^{c}	0.09 ± 0.01^{a}	0.14 ± 0.01^{bc}	0.13 ± 0.01^{abc}	0.15 ± 0.01^{ab}	0
MCV	**M**	67 ± 0.0^{a}	51.6 ± 1.5^{b}	52.33 ± 3.2^{b}	63.6 ± 3.2 ^{a}	66.6 ± 5.7 ^{a}	0
(fL)	**F**	50 ± 0.0^{b}	61 ± 0.0^{a}	63.9 ± 1.6^{a}	63.6 ± 3.2 ^{a}	61.6 ± 2.51^{a}	0

A – 0 day, B- Negative control, C- Positive control, D- *L. plantarum* MZ707748, E- *L. plantarum* MZ729681.
All values are represented as the (Mean ± S.D) of Wistar rats (male and female).
$^{a,b,c}P < 0.05$ compared with the control group by ANOVA followed by Tuckey test.

tive role against cadmium and mercury inhibitory effect and have the potential to be a beneficial supplement in rats' diets.

Zhao et al. (2020) reported that the typical serum concentrations of immunoglobulins A and G were significantly higher in the probiotic-treated group. The immunomodulatory effects of probiotics have been used to explain they are positive effects. Similar findings have been observed in the current research work. Paineau et al. (2008) used ($1x10^{10}$) CFU Bifidobacterium lactis and L. acidophilus strains and found that these microbes dramatically reduced serum IgG levels. The present study results revealed that the levels of IgA and IgG differed from control among male and female rat groups (Table 3).

The alanine transaminase (ALT) and aspartate transaminase (AST) levels were used to assess liver damage and the toxicity of

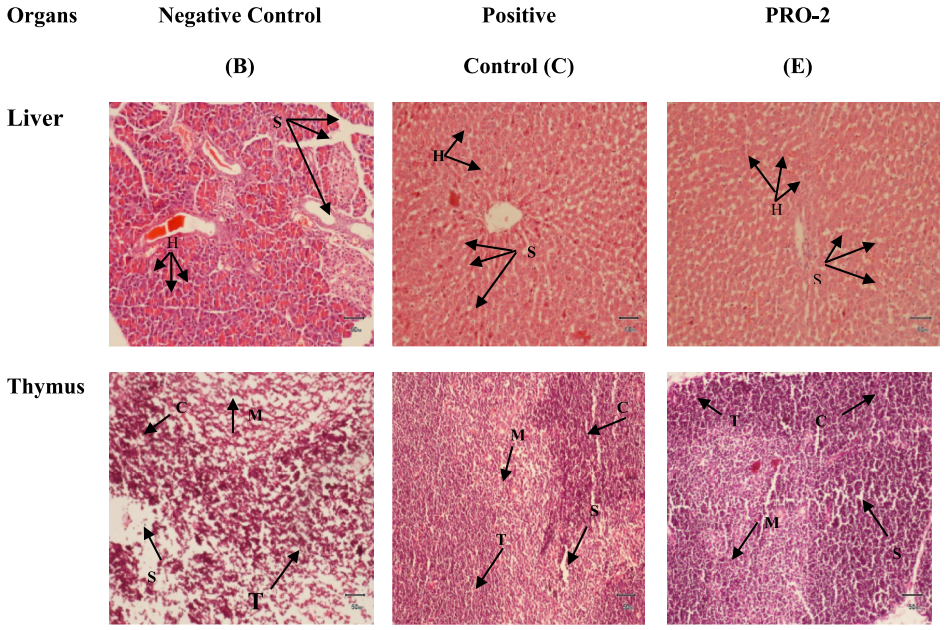

Fig. 6. Micrographs of Male Wistar rats Liver and Thymus at 10X; Negative control; C and E. Showing histological structures of Liver Hepatocytes (H), Sinusoids (S) and Thymus' Thymocytes (T), Cortex (C), Medulla (M), Spaces (S). No cellular or inflammatory changes were seen in the thymus and liver of male rats. Bar = 50 mm.

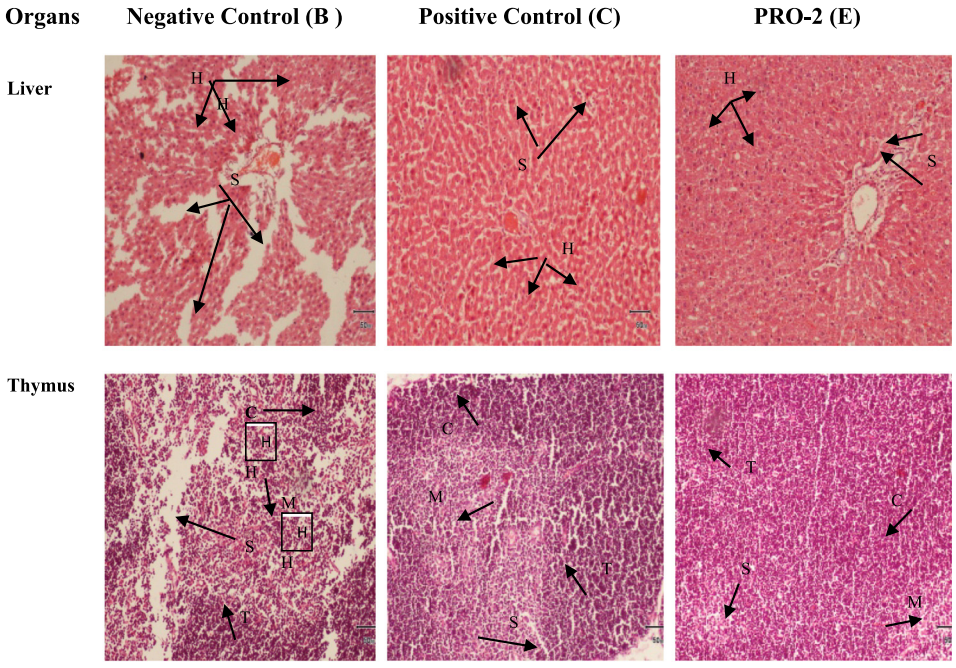

Fig. 7. Micrographs of Female Wistar rats Liver and Thymus at 10X; Negative control; C and E. Showing histological structures of Liver Hepatocytes (H), Sinusoids (S) and Thymus' Thymocytes (T), Cortex (C), Medulla (M), Spaces (S). No cellular or inflammatory changes were seen in the thymus and liver of female rats. Bar = 50 mm.

probiotics. The levels of IL-6, AST, and ALT in both genders were normal in the present study results. At the same time, no visibly significant difference between the non-treated and probiotics-treated groups was observed i.e. no liver damage occurred after probiotics treatment (Table 3). Nami et al. (2019) revealed that Lactobacillus plantarum YS5 strain isolated from homemade yogurt was found to lower AST, ALT, and cholesterol levels in rats. Javanshir et al. (2021) reported that *L. rhamnosus* GG alters innate and adaptive immune responses, particularly those directed against gastrointestinal infections, resulting in elevated serum IgG and secretory IgA levels that target intestinal pathogens like rotavirus. Other research has shown that *L. bulgaricus* OLL1073R-1 and its secretory polysaccharides boost immune system activity, which in turn stimulates NK cell activation. Therefore, using *L. bulgaricus* OLL1073R-1 or its products can prevent respiratory infections brought on by respiratory viruses like influenza (Makino et al., 2016; Javanshir et al., 2021).

The substance bilirubin metabolizes hemoglobin. Levels of bilirubin and creatinine signal the corresponding liver cell deterioration. Elevated bilirubin and creatinine levels may suggest illness of the liver, respectively (Dehkohneh et al., 2019). In the current study, male probiotics-treated groups had reduced bilirubin and creatinine levels. Still, female probiotics-treated groups had a modest increase that was not at a risk level (Table 3). Albumin keeps the blood in vessels without leaking. In the probiotics impact experiments, serum levels of globulins in both sexes were greater in probiotics-treated groups as compared to the control groups (Table 3). Probiotic research groups have reported similar findings working on animals to modify their immune function (Aminlari et al., 2019; Dehkohneh et al., 2019; Patra et al., 2022).

Complete blood count tests were performed to check the concentration of blood cells in male and female rats. Levels of HCT and WBC in male groups showed a remarkable difference, while there was a slight change among female groups. RBC and MCHC showed substantial change among female groups, while these values showed insignificant change among male groups (Table 4). de Carla Dias et al. (2020) reported that when Wistar albino rats were given oral supplements of *L. plantarum,* the concentration of Hb, PCV, and RBC increased. This result was attributed by Korcok et al. (2018) to the activation of the hematopoietic organs and some lactic acid bacteria, like Lactobacilli, which indirectly increase the availability of dietary iron through a series of processes, which include reducing intestinal pH. Obazelu et al. (2021) found that probiotics improved health conditions by raising hemoglobin levels, packed cell volume, and red blood cell count without causing harmful alterations to blood hematological parameters.

In the current investigation, male groups had a distinct change in HCT and WBC, while female groups had non-significant variations (Table 4). Similarly, RBC and MCHC levels varied significantly between the female and male groups (Table 4). de Carla Dias et al. (2020) reported that probiotics could improve health problems by boosting hemoglobin concentration, red cell count, and packed cell volume.

The thymus and liver were histopathologically examined in the present study to see any damage to the tissue organs' regular cellular structure. No abnormalities, such as normal structural losses, fibrosis, necrosis, inflammations, and atrophy, were found in any investigated organs in probiotics-administered groups (Figs. 6, 7). The significance of port specifications in liver histology cannot be emphasized enough. The core veins were found to be surrounded by typical hepatocytes. In the thymus, the Th-1 and Th-2 cells were shown to be higher in *Lactobacillus* groups (Baralic et al., 2020). Weight of rats' bodies and relative thymus and liver weight were measured. *L. plantarun* MZ707748*, L. plantarun* MZ729681, and *L. acidophilus*-14 raised these parameters but are insignificant as compared to the control. The present study results are the same as those reported by Chen et al. (2022).

5. Conclusion

Both isolated bacterial strains were recognized as LAB based on their microscopic, biochemical, and molecular characteristics. Probiotics strengthen the immune system by preventing the generation of pro-inflammatory cytokines and blocking numerous signaling pathways. Treated probiotic groups of rats may have a long-term beneficial effect on both genders but more on males than females. Commercially accessible probiotic strains were found to have similar immune system effects as laboratory-obtained probiotic bacterial strains. These findings indicate that the two Lactobacillus strains are safe and could be used as human probiotics. The current research may be beneficial for creating new probiotic products and may justify choosing a mix of probiotic strains. However, more clinical and preclinical research is required to assess the effectiveness of probiotic treatment.

6. Ethics approval

The Institutional Ethics Committee, GC University Lahore, has assessed and approved the existing research plan (Ref. No. GCU-IIB-117).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2023.04.023.

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