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Original article

Effect of probiotics on hematological parameters of male and female Wistar rats

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ABSTRACT

In the present study, the effect of probiotics on the hematology of Wistar rats was examined. Locally isolated *Lactobacillus plantarum* MZ707748 (Pro 1), *L. plantarum* MZ710117 (Pro 2), *Weisella confusa* MZ727611 (Pro 3), and *L. plantarum* MZ735961 (Pro 4) were used. One strain of probiotic, *L. acidophilus*-14 (Pro 5), was purchased commercially. Different groups were designed as G1, G2, G3, G4, and 5, G5/PC consisting only pro 5 and NC & 0 day were untreated. Different groups have different probiotics like G1 containing Pro 1 and Pro 2, G2 comprising Pro 3 and Pro 4, G3 containing Pro 2, Pro 3 and Pro 5, G4 having Pro 1–5, and G5 containing Pro 5. A complete count of blood, serum chemistry, fecal analysis, and histopathological examination of the thymus and liver were done. Statistical differences were seen in the complete blood count parameters ($p < 0.05$). No difference was observed in AST, ALT, bilirubin, albumin, IL-6, and IgA ($p > 0.05$) except for TP, creatinine, and globulin ($p < 0.05$). Fecal strains of probiotic groups were antibiotic-resistant. In males, *Lactobacillus helveticus* OQ152020, *Enterococcus lactis* OQ1519891, *E. faecium* OQ152017, *L. gasseri* OQ152017, and *E. lactis* OQ152019 were isolated from positive control, G1, G2, G3, and G4 respectively. In females, *Enterococcus* sp. OP800231, *Limosilactobacillus fermentum* OQ151985, *E. lactis* OP800267, *L. plantarum* OP800244, and *E. faecium* OQ151988 were isolated from positive control, G1, G2, G3 and G4, respectively. It was concluded that all probiotic strains were safe to use and had beneficial effects on the hematology of Wistar rats.

1. Introduction

It is observed that probiotics fortify the barrier of the intestine by increasing the tight junction (TJ) proteins, mucins, Paneth, and goblet cells. Besides immunity, they also play a role in maintaining the number of good bacteria in the gut and inhibiting the growth of bad microbes. The extended use of probiotics will not disturb the homeostasis of the intestine. People suffering from undernutrition or malnutrition, have significant effects on their immunity and intestine after the intake of probiotics. Dendritic cells and macrophages are the cells of the innate immune system that are activated to defend the body against pathogens. They slightly enhance the number of cells in lamina propria. The probiotics-killing activity has been seen in the macrophages of the spleen and peritoneum. These probiotics also activate cytokines that, in return, activate helper T cells. These cells produce the antibody IgG. Research suggests that the cell wall of microbes is responsible for the right functioning ability of systemic response of the immune system and mucosa. The immune system pathways activated by probiotics can be different (Liebl et al., 2009).

The microbes that are readily used as probiotics are the bacteria that produce lactic acid when undergo fermentation. They can be *Streptococcus, Lactobacillus, Bifidobacterium, Lactococcus, Leuconostoc,* and *Enterococcus* (Conley and Delacroix, 1987). These microbes inhabit the intestinal tract. Among all these bacteria, the largest group of bacteria is *Lactobacillus* (Macpherson and Uhr, 2004). These bacteria are commercially used in sauerkraut, wine, pickle,

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juices, beet, sausage, yogurt, and cheese. The lactic acid bacteria that are taken with diet are considered safe. This is because of their history in which they had a strong connection with diet and humans. *L. plantarum* WCFS1 is the very first of its kind whose whole genetic makeup was completely sequenced and this is the greatest genome among all other lactobacilli (Huijsdens et al., 2002).

Different probiotic formations have different efficacies depending on whether they consist of only one or multiple strains of probiotics (Aalaei et al., 2018). When compared with sole strain synthesis, the preparation of multiple probiotic strains can include the same species or genera more than a single strain or contain different genera. Sometimes, these probiotic combinations also include fungal species; *Saccharomyces* sp. (Timmerman et al., 2004). However, single probiotic strains have beneficial effects on alleviating or treating gastrointestinal-related diseases (Wilkins and Sequoia, 2017). Whereas it has been analyzed in-vitro that some probiotic combinations could portray better effects on inhibiting pathogens inhabiting the intestine (Chapman et al., 2013). Also, combinations of probiotics show a reduction in harmful chemical absorption in animals as well as humans because of the ability of their cell walls to absorb metals (Trinder et al., 2015). Therefore, their applications in detoxification therapy and biotechnology thus act as a supplement (Daisley et al., 2019). Since multiple strains of probiotics have better effects when compared with a single probiotic strain. But, all of these combinations did not show any superior effects when we compared them to the single strain (Chapman et al., 2013).

This investigation aimed to recognize more about the effects of orally given locally isolated *Lactobacillus* bacteria on rat hematological and serum variables. It was also aimed to inquire whether the efficiency of probiotics is affected by gender or not.

2. Material and methods

2.1. Test strains

Probiotics to be used were available in Cell and Molecular Biology Laboratory that were previously isolated from dairy and non-dairy products (Bibi et al. 2023). These strains included *Lactiplantibacillus plantarum* MZ707748, *L. plantarum* MZ710117, *L. plantarum* MZ735961, and *Weisella confusa* MZ727611. One strain of probiotic, *Lactobacillus acidophilus*-14, was purchased from Fazal Din Pharmacy, Lahore. All these probiotics were used in experimenting with their effects on the immune system of female Wistar rats.

2.2. Preparation of multi-strain probiotics and grouping

A total of 5 groups were designated as treated groups G1, G2, G3, G4, and G5. Group 1 (G1) consisted of *L. plantarum* MZ707748 and *L. plantarum* MZ710117. Group 2 (G2) constituted *L. plantarum MZ735961* and *W. confusa* MZ727611. Group 3 (G3) included *L. plantarum* MZ710117, *W. confusa* MZ727611, and *L. acidophilus.* Group 4 (G4) consisted of *L. plantarum* MZ707748, *L. plantarum* MZ710117, *L. plantarum* MZ735961 and, *W. confusa* MZ727611 and *L. acidophilus.* Group 5 (G5) was referred to as a positive control group. It consisted of *L. acidophilus.* All strains were mixed as per groups by 1:1 of CFU dilutions. Two untreated groups were referred to as negative control (NC) and 0 days.

2.3. Experimental model of Wistar rats

Wistar rats were purchased from the University of Veterinary and Animal Sciences, Lahore. Female rats weighed from 50 ± 6.66 g to 151 ± 2.72 g and male rats weighed from 91.66 ± 4.40 g to 248.86 ± 2.52 g. All male and female rats were allocated randomly to each group separately, with 3 Wistar rats in each group. Rats of each group were placed in different wooden cages. They were housed in the Rodents House of Government College University, Lahore. Environmental conditions were optimal; 12 h day, 12 h night cycle, 30 °C-37 °C, and 60–70% humidity. An experiment on both male and female Wistar rats was carried out under ethical certificate no. GCU-IIB-1065 issued by the ethical committee of Government College University, Lahore. After 1 week of acclimatization, rats were given multi-strain probiotics (4 mL) daily through gavage (Lollo et al., 2013). The dose was given for 5 weeks to each group. The negative control group was fed with normal chow and water, while 0 day was not housed during the whole experiment. Rats were euthanized by giving anesthesia (2 mL), and then they were dissected. Their blood was drawn for serum and complete blood count (CBC) analysis.

2.4. Fecal analysis

Fecal pellets were placed in phosphate buffer saline (3 pellets in 10 mL PBS). The mixture was centrifuged at 5000 rpm for 10 min. The supernatant was taken as a fecal extract. The fecal extract of rats from each group was plated on an MRS agar plate. These plates were grown overnight at 37 °C in the incubator. The colonies grown were then inoculated in MRS broth and grown overnight at 37 °C in the incubator. Antibiotic resistance was determined by the protocol of Haghshenas et al. (2017). Antibiotic discs used were penicillin (P-10) 10U, erythromycin (E-15) 15mcg, gentamicin (CN-120) 120mcg, cefixime (CFM-5) 5mcg, and ciprofloxacin (CIP-5) 5mcg.

2.5. Isolation of genomic DNA and 16S rRNA gene amplification

GeneJet genomic DNA purification kit (Cat # K0721) was used to isolate the DNA of bacteria from the feces of rats. For getting the bands of DNA, the gel was run via gel electrophoresis. 16S rRNA gene was further amplified using a polymerase chain reaction. For this purpose, universal primers RS3 (forward) and RS1 (reverse) were used (Bukhari and Shakoori, 2009). The sequences of these primers are given below.

Universal primers	Sequences
RS3 (forward)	5′-ACGGGCGGTGTGTA-3′
RS1 (reverse)	5′-AAACTCAAATGAATTGACGG-3′

The reaction mixture was made up to 25uL. Initial and final denaturation temperature was 95 and 94 °C for 5 and 2 min, respectively. Annealing was done at 52 °C for 90 sec. The initial and final extension was at 72 °C for 2 and 10 min, respectively. 35 cycles were completed by PCR then the gel was run. These PCR products were stored at −20 °C and sent for Sanger sequencing to Agro-Biotechnology Institute (ABI), Malaysia.

2.6. Statistical analysis

All the results were analyzed by Tukey's test ANOVA by SPSS (version 20). Alphabets based on differences in groups were found by MINITAB version 17. Graphs were made by GraphPad version 5.0.

3. Results

3.1. Antibiotic resistance

Antibiotic resistance of fecal isolated strains from each group was tested by the disc diffusion method. In males, 0 day was sus-

ceptible to all used antibiotics whereas G1, G2, G3, and G4 were resistant to all antibiotics. In females, negative control was susceptible to penicillin, gentamicin, and erythromycin and moderately susceptible to cefixime and ciprofloxacin. G3 was resistant to all antibiotics (Fig. S1; Table S1).

3.2. DNA isolation and genomic characterization

The genomic DNA of bacteria isolated from the feces of rats was isolated. After visualizing these bands on a UV illuminator, it was concluded that the size of the DNA bands was>10 kb. After confirming DNA bands, the 16S rRNA gene was amplified and the product size was 550 bp (Fig. S2a).

The sequences were submitted to the database of NCBI. In males, *Lactobacillus helveticus* OQ152020, *Enterococcus lactis* OQ1519891, *E. faecium* OQ152017, *L. gasseri* OQ152017, and *E. lactis* OQ152019 were isolated from positive control, G1, G2, G3, and G4. In females, *Escherichia* sp. OP800231, *Limosilactobacillus fermentum* OQ151985, *E. lactis* OP800267, *L. plantarum* OP800244, and *E. faecium* OQ151988 were isolated from positive control, G1, G2, G3, and G4. All these strains showed 97.42% to 100% similarity with their respective strains (Fig. S2b).

3.3. Complete blood count

Red blood cells were recorded in 10^6/uL. In males, it was highest in G4; 10.34 ± 0.87, and lowest in 0 day; 4.13 ± 2.04. While in females, the highest RBC count was observed in G3, 9.7 ± 0.5, and the lowest was seen in 0 day. White blood cells were recorded in 10^3/uL. The greatest WBC count was observed in G1; 15.56 ± 0.23 and G2; 12.2 ± 1.4 in male and female groups, respectively. The least WBCs were seen in G4 of male groups, 4.87 ± 0.35, and 0 day in female groups, 10.2 ± 0.2 (Fig. 1A1, A2).

Hemoglobin was recorded in g/dL. In males, it was recorded highest in G1; 16.03 ± 0.5, and lowest in negative control; 8.96 ± 3.5. In females, the highest hemoglobin was seen in G3, 16.1 ± 0.7, and lowest in negative control, 13.3 ± 0.8. Mean corpuscular hemoglobin was recorded on pg. In males, the highest MCH was seen in 0 day, 21.03 ± 1.2 whereas the lowest was observed in G4; 16.4 ± 0.6. In females, the highest MCH was seen in G1; 20.2 ± 0.3, and lowest in 0 day, 16.4 ± 0.2 (Fig. 1A1, A2).

Lymphocytes were recorded in percentage (%). Among male rats, 0 day had a higher lymphocyte percentage than the rest of the groups; 34.96 ± 2.39, and G2 had the lowest lymphocyte percentage; 78.56 ± 0.97. Mean corpuscular volume was recorded in fL. The highest MCV in male groups was found in 0 day, 65.6 ± 2.96, and the lowest was seen in G4; 44.4 ± 0.12. In females, the negative control group had the highest lymphocyte percentage; 63.7 ± 0.7 while the lowest was observed in 0 day, 40.7 ± 2.3.

Mean corpuscular hemoglobin concentration was recorded in g/dL. It was seen highest in 0 day of male groups; 30.6 ± 2.17 and lowest in G2; 39.5 ± 0.28. In females, G1 had the highest MCHC; 41.4 ± 0.7, and the least was observed in negative control; 32.9 ± 3.5. Hematocrit was recorded in percentage (%). In males,

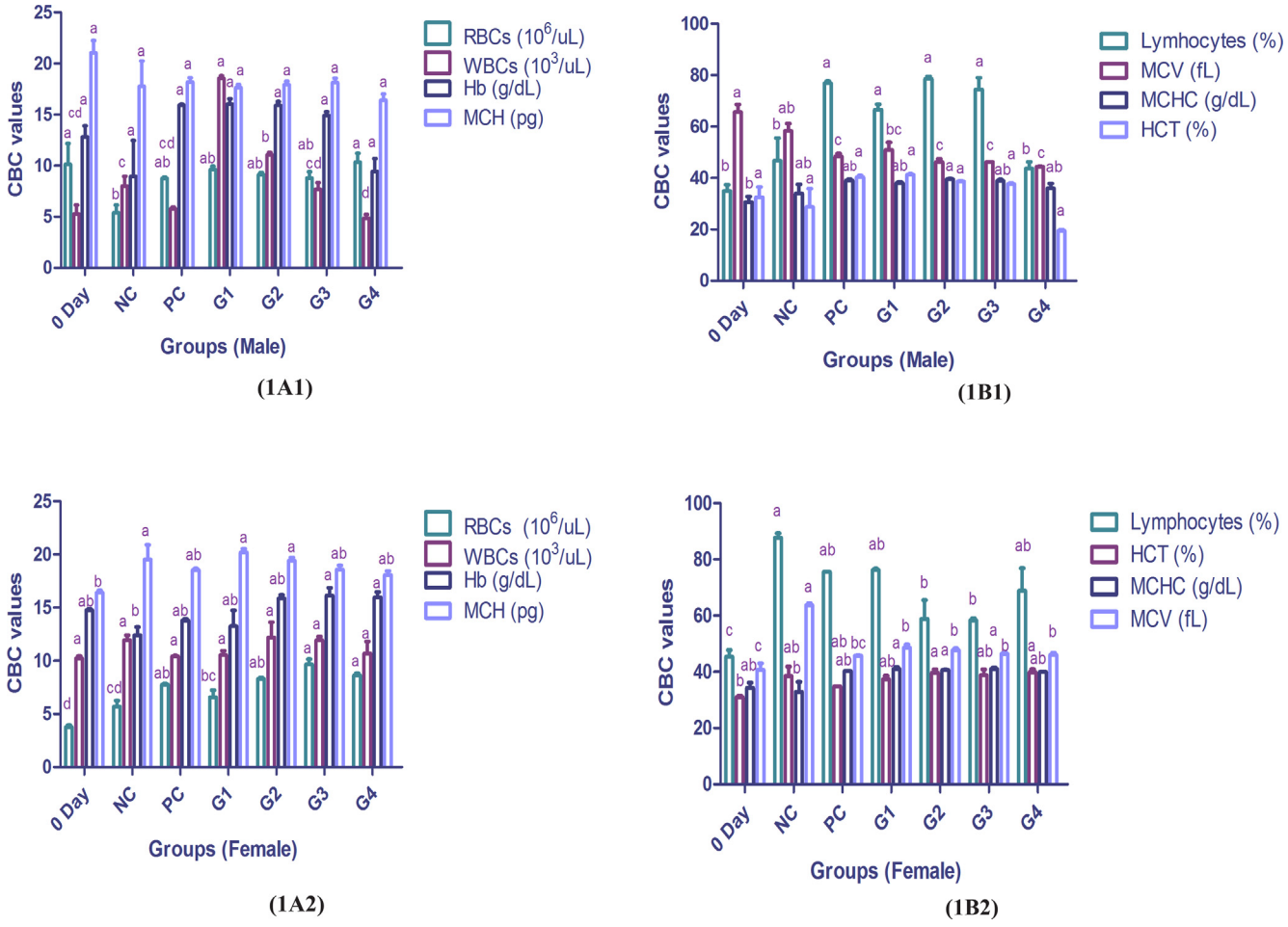

Fig. 1. A1, A2. Graph showing the mean of RBCs, WBCs, Hb, and MCH of male **(A1)** and female **(A2)** non-probiotic (0 day and NC) and probiotic (PC, G1, G2, G3 and G4) groups, error bars and alphabets on the basis of their differences. **B1, B2.** Graph showing the mean of lymphocytes, MCV, MCHC, and HCT of male **(B1)** and female **(B2)** non-probiotic (0 day and NC) and probiotic (PC, G1, G2, G3, and G4) groups, error bars and alphabets on the basis of their differences.

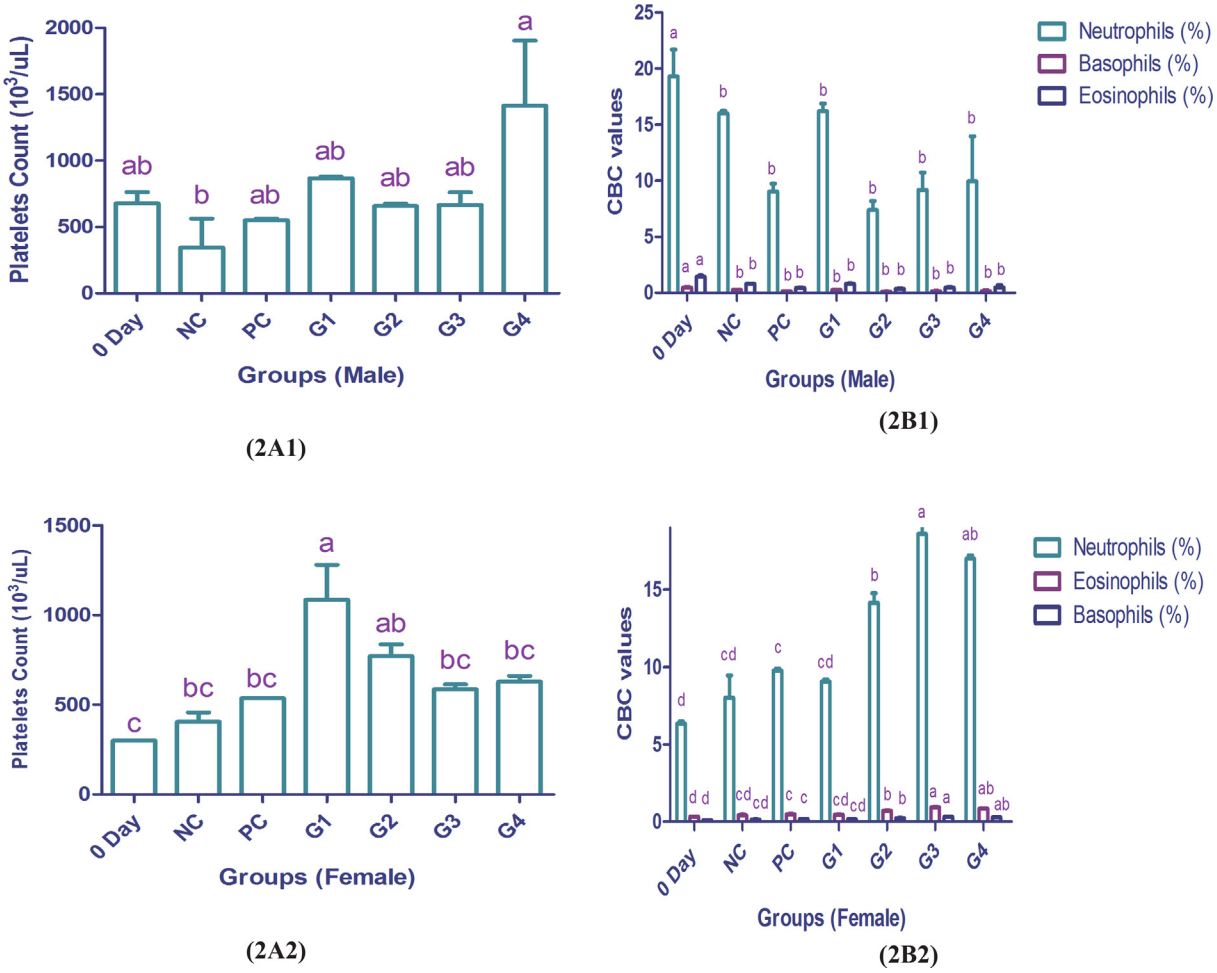

Fig. 2. A1, A2. Graph showing the mean of Platelets of male **(A1)** and female **(A2)** non-probiotic (0 day and NC) and probiotic (PC, G1, G2, G3, and G4) groups, error bars, and alphabets on the basis of their differences. B1, B2. Graph showing the mean of neutrophils, basophils, and eosinophils of male **(B1)** and female **(B2)** non-probiotic (0 day and NC) and probiotic (PC, G1, G2, G3, and G4) groups, error bars and alphabets on the basis of their differences.

G1 had the highest hematocrit, 41.3 ± 0.34 while the lowest in G4; was 29.4 ± 0.5 (1B1). In females, G4 had the highest HCT; 39.9 ± 1.1, and lowest in 0 day, 31 ± 0.6 (1B2).

Platelets were recorded in 10^3/uL. In males, the highest platelet count was seen in G4; 1413.7 ± 490, and the lowest was observed in negative control; 344 ± 218.4. In females, G1 seemed to have the highest platelet count, 1085 ± 196 whereas lowest in 0 day, 300 ± 0.96 (Fig. 2A1,A2).

Neutrophils were recorded in percentage. These seemed highest in 0 day, 28.6 ± 2.57, and lowest in positive control of males, 9.04 ± 0.96. In females, G3 had the highest neutrophil percentage; 18.6 ± 0.5, and lowest in 0 day, 6.3 ± 0.1. Eosinophils were recorded in percentage (%). In males, maximum eosinophils were found in 0 day, 1.43 ± 0.13, and the lowest was seen in positive control; 0.43 ± 0.03. In females, it was recorded highest in G3; 0.9 ± 0.1, and lowest in 0 day, 0.3 ± 0.01. Basophils were recorded in percentage (%). In males, it was highest in 0 day, 0.47 ± 0.04, and lowest in positive control; 0.14 ± 0.001. In females, it was seen highest in G3 and G4; 0.3 ± 0.001 and 0.03 ± 0.003 respectively whereas the lowest basophilic percentage was seen in 0 day and negative control; 0.1 ± 0.002 and 0.1 ± 0.02, respectively (Fig. 2B1,B2). All parameters of complete blood count in probiotics and non-probiotic groups of male and female Wistar rats seemed to be in the normal range. Combinations of probiotics positively impacted the hematology of rats. All results were significant in both genders (Table 1).

3.4. Serum analysis

Albumin was recorded in g/dl. In males, the lowest concentration was measured in G1,3.1 ± 0.5, and in females, the lowest concentration was seen in G3; 3.5 ± 0.36. Globulin was also recorded in g/dL. G3 showed the least globulin concentration in males; 3.23 ± 0.14 while in females, 0 day showed the least of it; 2.43 ± 0.28. Interleukin-6 (IL-6) was measured in pg/mL. The lowest IL-6 was determined in G2 of males, 15.8 ± 0.4, and in G3 of females, 15.3 ± 0.5. Total protein (TP) was calculated in g/dL. The lowest TP was seen in 0 day of males, 5.7 ± 0.05. While in females, it was lowest in positive control, 6.0 ± 0.05 (Table 2; Fig. 3A1,A2).

Immunoglobulin A was recorded in µg/ml. In males, the lowest IgA amount was measured in G2; 0.37 ± 0.01 whereas in females, the lowest concentration was determined in negative control. Creatinine was also recorded in mg/dL. The lowest creatinine was seen in G1 of male rats, 0.44 ± 0.04, and in G3 of females, 0.58 ± 0.01. Bilirubin was measured in mg/dL. In males, the lowest bilirubin was determined in G1. 0.25 ± 0.02 whereas in females, the lowest bilirubin was seen in negative control, 0.28 ± 0.9 (Fig. 3B1,B2).

Aspartate transaminase (AST) was measured in µL. The lowest AST was determined in 0 day, 263.6 ± 22.1, and 270 ± 25 in males and females, respectively. Alanine transaminase (ALT) was also recorded in µL. In males, the lowest ALT was determined in 0 day, 58.33 ± 3.52 (4A) while in females the lowest ALT was mea-

Table 1
Complete blood count parameters in different groups of male and female Wistar rats after administration of probiotics presented in Mean ± S.E.M. and their significance level (One Way ANOVA).

Parameters	Gender	0 Day	NC	PC	G1	G2	G3	G4	p value
RBCs (10^6/uL)	**M**	4.13± 2.04^{a}	5.40± 0.75^{b}	8.76± 0.12^{ab}	9.61± 0.34^{ab}	9.13± 0.2^{ab}	8.81± 0.59^{ab}	10.34± 0.87^{a}	0.03
	F	3.73± 0.2^{d}	5.74± 0.6 ^{cd}	7.72± 0.1^{ab}	6.61± 0.7^{bc}	8.33± 0.1^{ab}	9.74± 0.5^{a}	8.62± 0.2^{a}	0
WBCs (10^3/uL)	**M**	5.25± 0.90 ^{cd}	8.01± 0.95^{c}	5.78± 0.16 ^{cd}	15.56± 0.23^{a}	11.05± 0.24^{b}	7.69± 0.65 ^{cd}	4.87± 0.35^{d}	0
	F	10.23± 0.2^{a}	11.91± 0.5^{a}	10.42± 0.1^{a}	10.51± 0.4^{a}	12.24± 1.4^{a}	11.92± 0.3^{a}	10.73± 0.1^{a}	0.3
Platelets (10^3/uL)	**M**	678.01± 84.3^{ab}	344.00± 218.4^{b}	551.32± 10.4^{ab}	866.62± 11.6^{ab}	656.64± 19.7^{ab}	666.10± 95.2^{ab}	1413.71± 490^{a}	0.06
	F	300.00± 0.9^{c}	405.74± 52^{bc}	536.32± 1.2^{bc}	1085.00± 196^{a}	770.00± 66.8^{ab}	586.00± 27.8^{bc}	629.03± 32.7^{bc}	0
Lymphocytes (%)	**M**	34.96± 2.39^{b}	46.72± 8.84^{b}	76.81± 0.81^{a}	66.66± 2.02^{a}	78.56± 0.97^{a}	74.44± 4.58^{a}	43.67± 2.59^{b}	0
	F	59.74± 0.2^{c}	87.74± 1.5^{a}	75.62± 0.1^{ab}	76.24± 0.5^{ab}	58.93± 6.6^{b}	58.31± 0.7^{b}	68.94± 8^{ab}	0
Hb (g/dL)	**M**	12.83± 1.07^{a}	8.96± 3.5^{a}	15.91± 0.08^{a}	16.03± 0.5^{a}	15.91± 0.36^{a}	14.90± 0.37^{a}	9.43± 1.25^{a}	0.01
	F	14.72± 0.1^{ab}	13.33± 0.8^{b}	13.82± 0.2^{ab}	13.41± 0.5^{ab}	15.89± 0.3^{ab}	16.11± 0.7^{a}	16.00± 0.5^{a}	0.01
MCV (fL)	**M**	65.64± 2.96^{a}	58.33± 2.9^{ab}	48.41± 1.13^{c}	50.91± 2.91^{bc}	46.24± 1.24^{c}	46.12± 0.06^{c}	44.44± 0.12^{c}	0
	F	40.72± 2.3^{c}	63.74± 0.7^{a}	45.72± 0.1^{bc}	48.51± 1.2^{b}	47.71± 0.7^{b}	46.44± 0.4^{b}	46.13± 0.1^{b}	0
MCH (pg)	**M**	21.03± 1.2^{a}	17.72± 2.5^{a}	18.21± 0.41^{a}	17.64± 0.28^{a}	17.93± 0.34^{a}	18.13± 0.43^{a}	16.4± 0.6^{a}	0.22
	F	16.42± 0.2^{b}	19.51± 1.4^{a}	18.54± 0.1^{ab}	20.22± 0.3^{a}	19.43± 0.3^{a}	18.62± 0.4^{ab}	18.12± 0.4^{ab}	0.01
MCHC (g/dL)	**M**	30.61± 2.17^{b}	33.90± 3.6^{ab}	39.00± 0.57^{ab}	38.05± 0.52^{ab}	39.46± 0.28^{a}	38.81± 0.65^{ab}	35.92± 1.93^{ab}	0.03
	F	34.33± 1.8^{ab}	32.91± 3.5^{b}	40.33± 0.1^{ab}	41.42± 0.7^{a}	40.64± 0.1^{a}	41.01± 0.6^{a}	40.00± 0.1^{ab}	0.01
HCT (%)	**M**	35.89± 22.4^{a}	38.74± 7.15^{a}	40.22± 0.61^{a}	41.34± 0.34^{a}	38.71± 0.15^{a}	37.61± 0.38^{a}	29.43± 0.5^{a}	0.46
	F	30.98± 0.6^{b}	38.64± 3.1^{ab}	34.81± 0.1^{ab}	37.31± 1.3^{ab}	39.74± 1.2^{a}	38.81± 2^{ab}	39.88± 1.1^{a}	0.02
Neutrophils (%)	**M**	28.6± 2.57^{a}	16.00± 0.21^{b}	9.04± 0.69^{b}	16.23± 0.67^{b}	7.38± 0.81^{b}	9.18± 1.56^{b}	9.94± 4.03^{b}	0
	F	6.34± 0.1^{d}	8.00± 1.4 ^{cd}	9.81± 0.1^{c}	9.11± 0.1 ^{cd}	14.13± 0.6^{b}	18.62± 0.5^{a}	17.00± 0.2^{ab}	0
Eosinophil (%)	**M**	1.43± 0.13^{a}	0.84± 0.01^{b}	0.43± 0.03^{b}	0.81± 0.03^{b}	0.36± 0.04^{b}	0.45± 0.07^{b}	0.49± 0.2^{b}	0
	F	0.31± 0.01^{d}	0.42± 0.1 ^{cd}	0.53± 0.01^{c}	0.41± 0.01 ^{cd}	0.74± 0.03^{b}	0.91± 0.01^{a}	0.80± 0.01^{ab}	0
Basophils (%)	**M**	0.47± 0.04^{a}	0.26± 0.0^{b}	0.14± 0.01^{b}	0.27± 0.01^{b}	0.12± 0.01^{b}	0.15± 0.02^{b}	0.16± 0.06^{b}	0
	F	0.11± 0.002^{d}	0.11± 0.02 ^{cd}	0.21± 0.002^{c}	0.15± 0.002 ^{cd}	0.20± 0.01^{b}	0.31± 0.01^{a}	0.34± 0.003^{ab}	0

sured in positive control, 45.3 ± 4.8 (4B). All data of serum chemistry in males was non-significant except for IgA and total protein. All data of females was non-significant except for globulin, creatinine, and total protein. Results indicated that the usage of probiotics did not cause any harm to organs.

4. Discussion

The bacteria, at birth, are inoculated and various biome of microbes are developed when taken specific diet and feeding until the age of 3 to 5 years (Rodriguez et al., 2015). These microbes interrupt the synthesis of tissues and cells in the gut to boost immunity. Diet, aging, undiscerning medication use, or infection can pose challenges to disturb the microbes' makeup and the end products of fermentation. Irritable syndrome of the bowel (IBS), obesity, and inflammatory disease of the bowel (IBD) can be caused by the imbalance of the gut microbes (Yoshida et al., 2018; Kunnackal et al., 2018). In this case, a good approach to overcome this malfunction is to have a healthy diet that prompts the microbiota of the human gut. This diet consists of pre-and pro-biotics.

For the biochemical characterization of fecal bacteria of probiotics provided rats, an antibiotics susceptibility test was performed. The DNA of fecal bacteria was isolated to identify species. After the blood was drawn, the complete blood count and serum chemistry were analyzed to assess the immune system.

Groups G1-G5 were given probiotics whereas negative control and 0-day groups were not dosed with probiotics. Because of this, fecal bacteria isolated from probiotic groups had more concentration of probiotics than those not given probiotics. It was confirmed by biochemical tests (like catalase and gram staining). All strains isolated from rats provided with probiotics showed maximum resistance and/or moderate susceptibility to antibiotics like gentamicin, ciprofloxacin, penicillin, cefixime, and erythromycin. The fecal bacteria isolated from negative control and 0-day were susceptible or mildly susceptible to these antibiotics. These results are in good agreement with the results of Easson et al., 2022; Dobreva et al. 2022).

Table 2
Values of serum parameters in different groups of male and female Wistar rats after administration of probiotics presented in Mean ± S.E.M. and their significance level (One Way ANOVA).

Parameters	Gender	0 Day	NC	PC	G1	G2	G3	G4	p value
Albumin (g/dL)	**M**	3.30± 0.4^{a}	3.87± 0.4^{a}	4.00± 0.1^{a}	3.13± 0.5^{a}	3.90± 0.05^{a}	3.83± 0.12^{a}	4.17± 0.2^{a}	0.237
	F	4.53± 0.5^{a}	4.93± 0.5^{a}	4.56± 0.38^{a}	3.87± 0.2^{a}	3.53± 0.09^{a}	3.48± 0.36^{a}	4.26± 0.59^{a}	0.204
Globulin (g/dL)	**M**	3.43± 0.9^{a}	4.24± 0.17^{a}	3.39± 0.08^{a}	3.93± 0.17^{a}	3.57± 0.5^{a}	3.23± 0.14^{a}	4.63± 0.20^{a}	0.283
	F	2.43± 0.28^{a}	4.21± 0.4^{a}	3.82± 0.4^{a}	3.94± 0.17^{a}	2.56± 0.26^{a}	3.56± 0.23^{a}	3.97± 0.49^{a}	0.011
IgA (µg/mL)	**M**	0.45± 0.02^{b}	0.70± 0.00^{a}	0.43± 0.00^{b}	0.43± 0.00^{b}	0.37± 0.01^{b}	0.44± 0.03^{a}	0.62± 0.01^{b}	0.003
	F	0.23± 0.06^{a}	0.13± 0.03^{a}	0.33± 0.03^{a}	0.20± 0.05^{a}	0.26± 0.06^{a}	0.23± 0.03^{a}	0.13± 0.03^{a}	0.111
ALT (µL)	**M**	58.33± 3.52^{a}	68.0± 1.52^{a}	63.6± 2.60^{a}	71.00± 0.57^{a}	64.6± 2.84^{a}	61.00± 0.57^{a}	62.33± 5.60^{a}	0.124
	F	47.64± 5.4^{a}	54.31± 4.7^{a}	45.33± 4.8^{a}	56.64± 1.2 ^{a}	57.63± 3.8^{a}	47.69± 1.20 ^{a}	54.71± 2.18 ^{a}	0.173
AST (µL)	**M**	263.62± 22.1^{a}	368.62± 29.0^{a}	315.29± 30.9^{a}	264.0± 21.1^{a}	298.61± 4.6^{a}	320.56± 35.3^{a}	324.64± 13.8^{a}	0.054
	F	270.00± 25.0^{a}	368.71± 8.9^{a}	315.33± 30.9^{a}	277.67± 29.2^{a}	303.32± 1.8^{a}	304.00± 30.7^{a}	291.30± 28.1^{a}	0.186
Bilirubin (mg/dL)	**M**	0.30± 0.02^{a}	0.39± 0.01^{a}	0.33± 0.02^{a}	0.25± 0.02^{a}	0.27± 0.01^{a}	0.26± 0.03^{a}	0.29± 0.05^{a}	0.079
	F	0.37± 0.03^{a}	0.28± 0.9^{a}	0.29± 0.01^{a}	0.32± 0.02^{a}	0.39± 0.03^{a}	0.29± 0.01^{a}	0.35± 0.03^{a}	0.395
Creatinine (mg/dL)	**M**	0.50± 0.00^{a}	0.58± 0.04^{a}	0.55± 0.01^{a}	0.44± 0.04^{a}	0.51± 0.02^{a}	0.45± 0.03^{a}	0.50± 0.00^{a}	0.058
	F	0.61± 0.06^{a}	0.90± 0.05^{a}	0.81± 0.06^{a}	0.68± 0.01^{a}	0.78± 0.07^{a}	0.58± 0.01^{a}	1.03± 0.09^{a}	0.001
IL-6 (pg/mL)	**M**	17.01± 0.14^{a}	18.53± 0.43^{a}	16.64± 0.6^{a}	16.80± 0.3^{a}	15.8± 0.4^{a}	16.11± 0.18^{a}	17.00± 0.6^{a}	0.018
	F	16.44± 0.5^{a}	15.73± 1.1^{a}	16.31± 0.3^{a}	15.62± 0.4^{a}	17.22± 0.3^{a}	15.31± 0.5^{a}	16.47± 0.6^{a}	0.324
Total Protein (g/dL)	**M**	5.70± 0.05^{d}	7.83± 0.08^{b}	7.02± 0.20^{c}	7.56± 0.08^{bc}	7.26± 0.14^{bc}	7.66± 0.12^{b}	9.73± 0.12^{a}	0.001
	F	6.80± 0.43^{ab}	8.30± 0.65^{a}	6.00± 0.05^{b}	8.33± 0.20^{a}	6.56± 0.29^{b}	6.33± 0.08^{b}	6.66± 0.33^{ab}	0.001

-Alphabets on the values as superscripts show statistical difference between groups.

Serum and hematological data are crucial indicators to determine toxic effects caused by cures. The CBC parameters are used to determine infections and inflammation whereas serum chemistry detects organ-associated problems. Values of RBCs were significant in untreated and treated groups. Groups with the synergy of probiotics contained more RBC concentrations than those that were not given probiotics. Testosterone in males is linked with erythropoiesis. Genetic differences were seen in the gene and receptors of erythropoietin. It demonstrates higher RBCs and HCT in males compared to females (Zeng et al. 2001). WBCs also showed significant results in groups. The lowest of WBCs were observed in G4 and 0 day in males and females respectively. These results were in accordance with Negi et al. (2022), Ou et al. (2022), and Sujaya et al. (2022).

The results of hemoglobin in groups were also significant. In males, Hb was highest in G1, and in females, it was highest in G3 whereas lowest in negative control. Platelets are the coagulating agents for the incidence of wounds. Greater concentrations of platelets may lead to coagulation of blood in the vessels whereas deficiency of them causes a problem in wound healing. All groups showed normal values of platelets. G1 had maximum platelets and the least platelet count was seen in 0 day in females while G4 had the highest platelet count in males. It was because *Lactobacilli* utilize the sugar that the vaginal cells store under the influence of estrogen (Kaur et al., 2020). Hormones affect hematological parameters (Grau et al., 2018). Females having greater estradiol levels have higher platelets (Daly, 2011). However, it can be said that different probiotic combinations did not show significant results in females compared to male rats. These results were similar to the findings of Sujaya et al. (2022 and Ou et al. (2022).

Lymphocytes are the cells to defend the body against invaders. These can be T and B cells and NK cells. Probiotics groups, 0 day, and negative control had a normal range of lymphocytes which means the body is not having any infection with or without the probiotic ingestion. However, the results showed differences in groups. Neutrophils are the granulocytes that help in immune functions. In males, they were maximum in 0 day whereas in females, they were highest in G3. In females, G3 showed the maximum ratio of neutrophils to lymphocytes, and in males, it was highest in positive control. These results were in accordance with Sujaya et al. (2022).

MCH is the mean of hemoglobin present in the blood. In males, it was found greater in 0 day whereas, in females, it was greater in G1. Results showed significance. MCHC is the indicator to detect the hemoglobin amount. It was recorded highest in 0 day and G1 in males and females respectively. The study suggests that gender has no significant effect on MCHC levels. However, the difference in MCHC in males and females could be because of a change in the activity of either gender (Rashid et al., 2016). These results were according to Darwish et al. (2022), Sujaya et al. (2022), and Ou et al. (2022).

MCV is the parameter for the size measurement of RBCs. If MCV is lower, that means the cell size is small. All values in groups were normal in range. Negative control and 0 day had the highest MCV level in males and females respectively. All probiotic combination groups were optimum in size for red blood cells. Results in groups

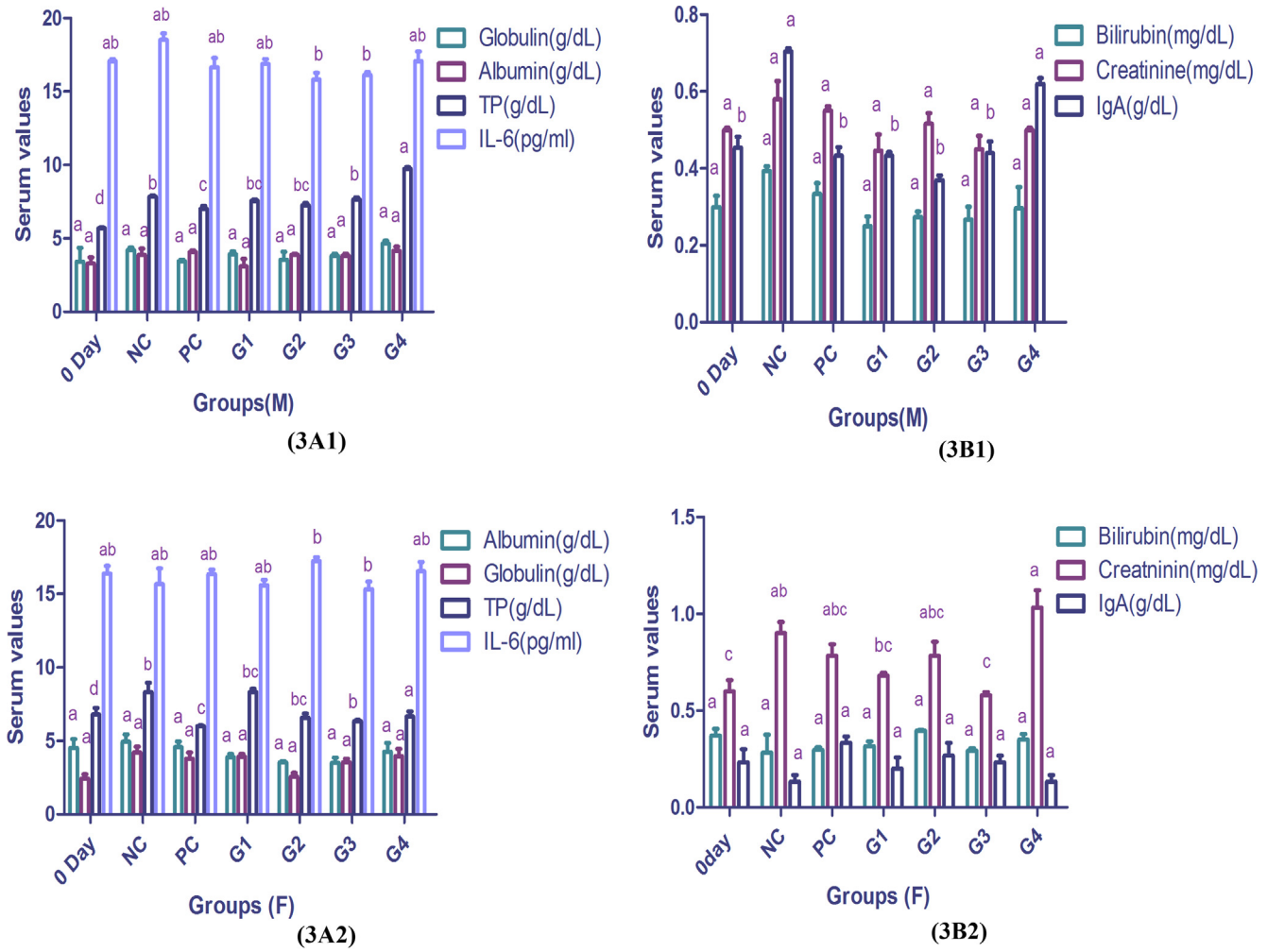

Fig. 3. A1, A2. Graph showing the mean of globulin, albumin, total protein (TP), and interleukin-6 (IL-6) of male **(A1)** and female **(A2)** non-probiotic (0 day and NC) and probiotic (PC, G1, G2, G3, and G4) groups, error bars and alphabets on the basis of their differences. B1, B2.Graph showing the mean of bilirubin, creatinine, and immunoglobulin A (IgA) of male **(B1)** and female **(B2)** non-probiotic (0 day and NC) and probiotic (PC, G1, G2, G3, and G4) groups, error bars and alphabets on the basis of their differences.

were significant. HCT refers to the RBCs' amount. These values seemed to be highest in G1 and G3 in males and females respectively. Since hematocrit has a direct link with RBCs level, probiotic combinations affected both genders differently. These results were in accordance with Negi et al. (2022), and Darwish et al. (2022).

Basophils and eosinophils defend the body against parasites, allergens, and other pathogens. These were highest in PC and G3 in males and females respectively. The lowest levels were seen in 0 day in both genders. These results were in good agreement with Ou et al. (2022), and Darwish et al. (2022).

Serum results indicate the health of respective organs. ALT is the enzyme of the liver that breaks food into energy. Blood contains a lower ALT level produced by the liver but if its concentration is higher, it indicates the damage in liver. Fortunately, ALT levels were in the range of normal in all groups either they were probiotic groups or non-probiotic groups. Similarly, AST is present in the catalytic reactions of glutamate and aspartate. There was no difference seen in the AST levels of untreated and treated groups. These results were according to Liao et al. (2022), and Easson et al. (2022).

Immunoglobulin A and interleukin-6 are the factors involved in the immune system acting against antigens. IgA levels were lower in G2 and NC in males and females. IL-6 was found to be lower in G2 and G3 in the respective genders. No difference was seen in groups. These results were according to Easson et al. (2022), and Liao et al. (2022). Albumin keeps the blood in vessels without leaking. Albumin was recorded least in G1 and G3 in males and females. Globulin helps in the movement of nutrients and fighting infection. Least globulin was found in G3 and 0 day in males and females. The differences in these groups were<0.05. The results were in good agreement with the findings of Liao et al. (2022).

Bilirubin is responsible for metabolizing hemoglobin. A higher bilirubin level demonstrates jaundice. G1 and NC showed the least bilirubin in males and females. No difference was seen. Creatinine was recorded as lowest in G1 and G3 in males and females. These results were similar to those of Sarwar et al. (2022), and Easson et al. (2022). Total protein is the parameter to detect proteins in the body. Greater or lower protein level indicates infection in the organs. The highest TP level was measured in G4 and G1, and the lowest was determined in 0 day PC in males and females, respectively. However, all values were in the normal range, suggesting no kidney, liver, or any other organ damage. These results were according to Liao et al. (2022), and Darwish et al. (2022).

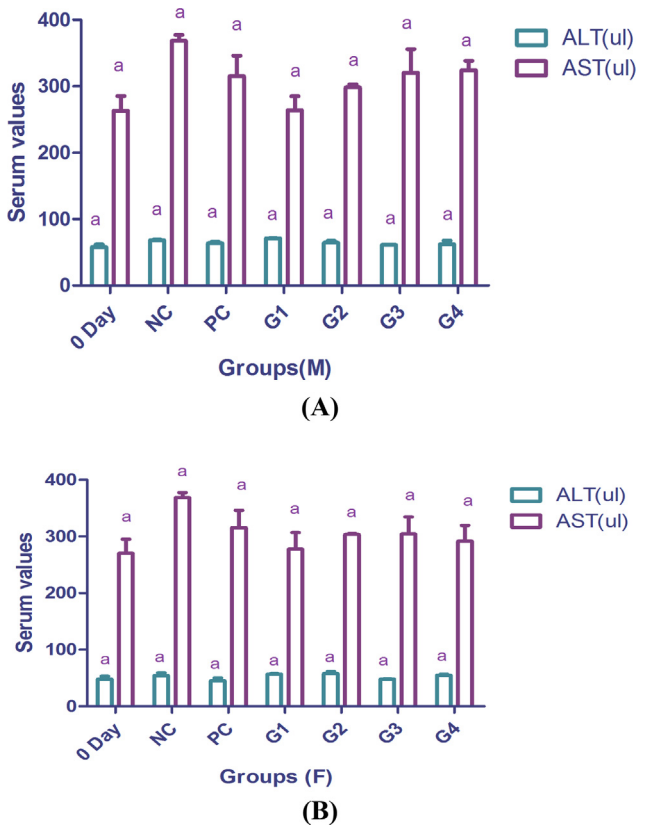

Fig. 4. Graph showing the mean of alanine transaminase (ALT) and aspartate transaminase (AST) of male **(A)** and female **(B)** non-probiotic (0 day and NC) and probiotic (PC, G1, G2, G3, and G4) groups, error bars and alphabets on the basis of their differences.

5. Conclusion

In conclusion, all probiotic combinations significantly affected rats' hematology and clinical chemistry, but the best combination of probiotics was *L. plantarum, L. acidophilus,* and *W. confuse* that was given to G3 of both male and female rats. Different groups based on probiotics were designed. A complete count of blood, serum chemistry, fecal analysis, and statistical differences were seen in the complete blood count parameters ($p < 0.05$). Histopathological examination of the liver and thymus was also performed. No difference was seen in AST, ALT, bilirubin, albumin, IL-6, and IgA ($p > 0.05$) except TP, creatinine, and globulin ($p < 0.05$). Fecal probiotic strains were found antibiotic-resistant. *Lactobacillus helveticus* OQ152020, *Enterococcus lactis* OQ1519891, *E. faecium* OQ152017, *L. gasseri* OQ152017, and *E. lactis* OQ152019 from males and *Enterococcus* sp. OP800231, *Limosilactobacillus fermentum* OQ151985, *E. lactis* OP800267, *L. plantarum* OP800244, and *E. faecium* OQ151988 from females were isolated from positive control, G1, G2, G3, and G4, respectively. Thus, it can be concluded that combinations of probiotics can have a good impact on hematology and are safe to use. This study showed that different probiotic combinations had different effects on the hematological parameters of Wistar rats. Further molecular mechanisms of action of these probiotics' combinations are needed to be studied.

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Ethical statement

This study was conducted under ethical certificate no. GCU-IIB-1065 issued by the ethical committee of Government College University, Lahore.

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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103794.

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