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Research Article

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The Effect of Azolla Feeding on Immunological Traits, Gut Microbiota and Gene Expression of Broiler Chicks

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Abstract The target of present research was to determine how different doses of Azolla and probiotics affect cecal in intestinal microbiota, gene expression and immune competence traits of Indian River (IR) chicks. The study on 300 IR chicks, which was randomly divided into ten groups each with three replicates of 10 birds. The first (T1) were fed diet without Azolla while, Azolla 5% (T2), 10% (T3), 15% (T4), 20% (T5), however birds in group (T6) fed diet without Azolla and drink water with 10^8 *Enterococcus faecalis*, Azolla 5% with 10^8 *Ent* (T7), 10% with10^8 *Ent* (T8), 15% with10^8 *Ent* (T9) and 20% with10^8 *Ent* (T10). The experiment lasted for 35 days then all animals were slaughtered. Revealed data demonstrated that total count bacterial in pathogenic and probiotics bacteria that total clostridium and Escherichia coli (E. coli) and probiotics that lactic acid, bacillus subtilis and Enterocooi, gene expression that immunity from IL6 , IL4 and MUC2 and antioxidants from INOS , SOD1 and LXRA. As a result, in improve beneficial microflora and reduce pathogens in caecum, data in IL6 were found to be elevated in birds and significantly increased in Azolla 20% + Pro group compared to control. IL4 and MUC2 showed insignificant expression compared with control. Indeed, antioxidant with the INOS significantly in birds Azolla 20% group compared to control. SOD1 and LXRA insignificant compared with control. Based on the study, using Azolla as replacement level of 5% of soy protein and without probiotic *(Enterococcus faecails)* improve beneficial microflora and reduce pathogens in caecum. and suggest of Azolla and probiotics in the diets of broiler chicks has a varying effect on the gene expression of immunity-related genes and antioxidant-related genes in the cecal tissue.

Keywords Azolla pinnata, immune function, gene expression, caecal microbiome

Introduction

As a result of its antifungal activity against numerous fungal infections, the aquatic fern genus Azolla has also demonstrated antifungal qualities and has been utilized in traditional medicine. Azolla has been thought of as a feed supplement for cattle in addition to its possible use as an antifungal agent. Azolla is rich in protein and can provide animals with essential nutrients, especially in regions with scarce feed supplies (Selvaraj *et al.,* 2013). Azolla can increase the development rates and feed efficiency of fish, pigs, and poultry when added to their regular diets. Moreover, because of its capacity to fix atmospheric nitrogen, azolla has been utilized as a biofertilizer (Selvaraj *et al.,* 2014). Azolla can serve as a substitute supply of nitrogen, lessening the demand for synthetic fertilisers as

interest in organic farming grows (Jumadi *et al.*, 2014). Azolla has also been investigated for its potential to enhance livestock's immunological response. According to studies of Ampode and Eladia (2021); and Ampode and Lagua (2021), feeding hens Azolla can boost their immune system activity, enhancing their disease resistance. Azolla also includes anti-inflammatory and antioxidant chemicals that can aid in lowering inflammation and oxidative stress in animals. Animals raised in stressful environments, such as those with high temperatures or insufficient nutrition, may benefit most from these qualities (Chichilichi *et al*., 2015).

Diet, environment, and genetics are just a few of the variables that might have an impact on the gut microbiota's makeup. Azolla has been shown to have positive impacts on nutritional absorption and intestinal health in grill chicks. Broiler chicks fed Azolla had higher concentrations of Lactobacillus and Bifidobacterium in their gut microbiota, which can improve gut health and boost immunological response (Samad *et al.*, 2020). Azolla may boost the digestion and utilization of dietary nutrients, which would result in lower feed costs and improved growth performance in livestock. This possibility is supported by the increase in beneficial bacteria and the changes in gene expression associated to nutrient metabolism (Yadav and Jha, 2019).

Abd El-Ghany (2020) found discovered that feeding Azolla to chicks significantly altered their gene expression in ways that affected their immune system, stress response, and food metabolism. The increased expression of genes involved in food metabolism shows that azolla may enhance the chicks' absorption of dietary nutrients. The overexpression of genes involved in stress response and immunological function may suggest that azolla has a beneficial impact on the chicks' immune systems and stress tolerance. Mishra *et al.* (2016) studied that, adds to the body of evidence supporting the potential advantages of Azolla as a livestock feed addition. The ability of Azolla to improve gut health, immune function, and nutrient utilization, as well as its high protein content and ability to grow rapidly make it an attractive candidate for further research into its use in livestock nutrition.

The aim of the work investigates the effect of feeding different levels of Azolla and probiotic on cecal in intestinal microbiota, gene expression and immune competence traits of Indian River (IR) chicks.

Materials and Methods

The experimental work of the present study was carried out in the privet farm at Moshtohor and laboratories belonging to regional center for food & feed with the cooperation of Departments of animal production, faculty of agriculture, benha university. It was aimed to investigate the effect of feeding different levels of Azolla and probiotic on cecal in intestinal microbiota, gene expression and immune competence traits of Indian River (IR) chicks.

Experiment treatments and birds:

Three hundred one-day-old male broiler Arbor Acres chicks were weighed equally and randomly divided and distributed in ten dietary treatments groups, consists of 30 chicks distributed in three replicated pens, with 10 chicks in each. The chicks were maintained on a 24 hours consistent lighting schedule and proper ventilation was ensured. The birds in the control group (T1) were fed diet without Azolla while, Azolla 5% (T2), 10% (T3), 15% (T4), 20% (T5), however birds in group 6 (T6) fed diet without Azolla and drink water with 10^8 *Enterococcus faecalis*, from hatching day to the end of experimental work, Azolla 5% with 10^8 *Enterococcus faecalis* (T7), 10% with10^8 *Enterococcus faecalis* (T8), 15% with10^8 *Enterococcus faecalis* (T9) and 20% with10^8 *Enterococcus faecalis* (T10). The experiment lasted for 35 days then all animals were slaughtered. The chicks in all treatments were kept under similar hygienic and environmental conditions, vaccinated against Newcastle and Gumboro diseases, housed in the floor with wire border under continuous fluorescent lighting (10 watt/m^2), and provided on un-medicated corn soybean-based meal diet (containing no added antibiotics, coccidiostats, or growth promoters) and water *ad libitum.*

Experimental diets:

Table 1: Calculated chemicals composition of starter diet and diet ingredients

Feed stuff	Starter				
	F1	**F2**	**F3**	**F4**	**F5**
Soybean meal %	21.8	19	19	17	16
Maize %	46.5	44.3	46.8	47	46.8
Corn bran %	10	9	5	3	2
Azolla %	0	5	10	15	20
Bone meal %	0	1	0	0.8	0
Salt %	1	1	1	1	1
Oil %	5	5	5	5	5
*Premix %	1	1	1	1	1
Concentrated (52) %	14.5	14.5	12	10	8
Lysine %	0.1	0.1	0.1	0.1	0.1
Methionine %	0.1	0.1	0.1	0.1	0.1
Total	100	100	100	100	100
Ingredient					
Crude protein (%)	22.77	23.10	23.22	23.00	23.07
ME (kcal/kg)	3284	3251	3259	3262	3240

Where: F1: control, F2: 5%Azolla, F3: 10% Azolla, F4: 15% Azolla, F5:20%Azolla.

Table 2: Calculated chemicals composition of grower diet and diet ingredients

Feed stuff	Grower				
	F1	**F2**	**F3**	**F4**	**F5**
Soybean meal %	19.5	18.5	13	10	7
Maize %	47.3	43.3	51	43.3	42
Corn bran %	15	17	9.8	17	17
Azolla %	0	5	10	15	20
Bone meal %	1	2	0	0.5	0.8
Salt %	1	1	1	1	1
Oil %	5	5	5	5	5
*Premix %	1	1	1	1	1
Concentrated (52) %	10	7	9	7	6
Lysine %	0.1	0.1	0.1	0.1	0.1
Methionine %	0.1	0.1	0.1	0.1	0.1
Total	100	100	100	100	100
Ingredient					
Crude protein (%)	20.30	20.15	20.11	20.02	19.94
ME (kcal/kg)	3287	3240	3334	3325	3320

Where: F1: control, F2: 5%Azolla, F3: 10% Azolla, F4: 15% Azolla, F5:20%Azolla.

Probiotic strains:

Strains of *Enterococcus Faecalis* used in this study. These strains were isolated, purified, identified, stored and kindly supplied by Food Safety and Biotechnology Laboratory, regional center for food and feed, A.R.C., Giza, Egypt. (Tables 4 and 5):

Azolla pinnata

Scientific classification of *Azolla*:

Kingdom: Plantae
Order: Salviniales
Family: Salviniaceae
Genus: Azolla

Chemical composition of Azolla meal (AZM):

Azolla meal containing **Nutrient** 12.7% fiber, 21.4% protein, 16.2% Ash, 2.7% Ether extract, 47% NFE, **Cell wall fraction**: 36.88% Neutral detergent fiber, 47.08% Acid detergent fiber, 10.2% Hemicellulose, 12.76% Cellulose, 28.24% Cellulose, Minerals: 1.16% Calcium, 1.29% Total phosphorus, 1.25 Potassium, 0.35 Magnesium, **Trace minerals**: 174.42 ppm DM Manganese, 87.59 ppm DM Zinc, 16.74 PPM DM Copper, 755.73 PPM DM Iron, 23.79 PPM DM Sodium.

Table 3: Composition of Azolla meal amino acid

Amino acids	%DM	g/100g Protein	Chemical score (%)
Methionine	0.34	1.59	45.4
Lysine	0.98	4.58	130.9
Threonine	0.87	4.07	116.3
Cystine	0.18	0.84	24.0
Tryptophan	0.39	1.82	52.0
Isoleucine	0.93	4.35	124.3
Arginine	1.15	5.37	153.4
Phenylalanine	1.01	4.72	134.9
Tyrosine	0.68	3.18	90.9
Leucine	1.65	7.71	220.3
Serine	0.90	4.21	120.3
Valine	1.18	5.51	157.4
Glycine	1.00	4.60	131.4

Media, reagents, chemicals and kits used:

Determination of caecal colonization:

Caecal material was serially diluted from initial 10-1 to 10-8 in sterile saline solution in a ratio of 1:9 and plated on Violet Red Bile Agar (VRB, Biolife), De Man, Rogosa and Sharpe agar (MRS, Biolife), Slanetz Bartley agar (SBA, Biolife), and Clostridium Agar (Biolife), which were used for the isolation and enumeration of E. The total number of grown colonies was calculated, and the collected colonies underwent biochemical tests. Using biochemical tests (API 20E, API Strep, Bio Merieux), all bacteria were identified. Also employed in the counting of anerobic bacteria was Clostridial agar. Prior to initial inspection, anaerobic incubation was performed for a minimum of 7 days in anaerobic jars (Oxoid). Anaerobic conditions were created using anaerogen (Oxoid), and methyl blue strips were used as an oxidation reduction indicator to monitor them. After seven days of incubation at 37 °C. Size, form, edge, profile, color, opacity, hemolysis, fluorescence, pigment, and pitting characteristics were all included in the colony description. Double layers of MRS agar were used to count the Bacillus subtilis and Lactobacillus species. The plates were kept in a microaerophilic environment for two days at 37 °C. The counting of E. coli used multiple layers of VRB agar. The plates were kept in a microaerophilic environment for 24-48 hours at 44 ºC. According to Ahmed *et al.* (2020).

Determination of pH in the caecal contents:

After the end of experiment 35 days of age, 4 chicks from each treatment within each group were slaughtered by cervical dislocation. Caecal contents were aseptically removed, and 0.2 g was suspended in 0.8 mL of sterile glass

distilled water. One mL of distilled water was added to the suspension then measure by using thermo Orion pH meter after calibration with (pH 4.0, 7.0 and 10.0).

Table 4: Isolation and identification of probiotics

Media	Ingredients per liter	pH
MRS Agar (Biolife, 1991)*	Peptone 10 g, Beef Extract 10g, Yeast Extract 5g Glucose 20 g, Dipotassium Hydrogen Phosphate (K_2HPO_4) 2, Sodium Acetate 5g Ammonium Citrate 2g, Magnesium Sulphate ($MgSO_4$) 0.2 g, Magnesium Sulphate ($MgSO_4$) 0.05, Agar 15 g, Agar Bios LL 20 g Tween80 mL 1	pH 6.4 ±0.2 at 25ºC
SLANETZ BARTLEY AGAR (SBA) (Biolife, 1991)*	Trypotse 10 g, Glucose 2 g, Yeast Extract 5 g, Sodium Azide (NAN_3) 0.4g, Potassium Phosphate Bibasic (K_2HPO_4) 4 g, TTC 0.1, Agar 10 g	pH 7.2±0.1at 25ºC
Clostridium Agar (Biolife, 1991)*	Beef Extract 10g, Yeast Extract 3.0g, Tryptone 10 g, D-Glucose 5g, Sodium Starch 1g, sodium Acetate 0.3 g, Sodium Chloride 5 g, Cysteine HCl 0.5g, Agar Bios LL 15 g	pH 6.8 ±0.2 at 25ºC
VLOLET RED BILE AGAR (V.R.B) (Biolife, 1991)*	Yeast Extract 3g, Peptone 7g, Bile Salts No3 1.5g, Sodium Chloride 5g, Lactose 10g , Crystal Violet 0.002g, Agar 10g, Neutral Reo 0.03.	pH 7.4 ±0.2 at 25ºC

*The required quantity was prepared as mentioned by the manufacturer.

Gene expression:

RNA Extraction:

Total RNA was extracted from tissues samples from the caecum and liver sections using Trizol Reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) following the manufacturer's protocol.

Lyse samples and separate phases:

Samples should be lysed and homogenized in TRIzol™ Reagent in accordance with the starting material. Utilizing a homogenizer, add 1 mL of TRIzol™ Reagent per 50–100 mg of tissue to the sample. To allow the nucleoproteins complex to completely dissociate, incubate for 5 minutes. For each 1 mL of the TRIzol™ Reagent used for lysis, add 0.2 mL of chloroform before tightly capping the tube. For two to three minutes. Centrifuge the sample at 12,000 g for 15 minutes at 4 °C. The mixture divides into an upper colorless aqueous phase and a lower red phenol-chloroform, interphase, and phase. By tilting the tube at 45°C and pipetting the solution out, you can transfer the aqueous phase containing the RNA to a fresh tube.

RNA assessment:

Using a (NanoDrop 1000, USA) spectrophotometer, it is essential to evaluate the RNA content and purity in extracted samples. The concentration of nucleic acids can be precisely measured using the absorbance at 260 nm, as well as at 280 and 230 nm.

Reverse transcription:

Reverse transcription, which uses the extracted RNA as a template to create cDNA, comes after RNA extraction and quality checks. Utilizing the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific / Ferments) in accordance with the manufacturer's instructions, the reverse transcriptase enzyme uses the RNA template and

short-sequence primers to direct the synthesis of the first strand cDNA, which is then utilized as a template for the qPCR reaction.

Quantitative Real-Time PCR:

The Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific / Fermentas, Vilnius, Lithuania) was used to create complementary DNA (cDNA) for quantitative reverse transcription PCR (RT-qPCR) in accordance with the manufacturer's instructions. The obtained cDNA was kept at 20 C after being diluted to 70 L working solutions. A total of two technical replicates of each RT-qPCR reaction were performed. MUC2 mucin, IL-4 interleukin 4, IL-6 interleukin 6, iNOS nitric oxide synthase, SOD1 super oxide maltase, LXRa liver x receptor, and beta-actin reference genes were included in the cytokine gene panel. These genes were used to normalize the data. Table 5 lists the oligonucleotide sequences of the primers.

RT-qPCR reactions were conducted with a total volume of 20 µL. The reaction mixture included Maxima SYBR Green qPCR Master Mix (Thermo Scientific/Fermentas, Vilnius, Lithuania), one µM of each primer and 2µL of diluted cDNA (70 ng/µl). Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The thermal program included a step of initial denaturation (15 min at 95 ◦C), followed by 40 cycles of denaturation (10 s at 95 ◦C), annealing (15 s at 58 ◦C), and extension (30 s at 72 ◦C). Fluorescence was measured at the end of each extension step. After completing the thermal program, the melting curve was generated, which indicated amplification specificity. The thermal program for the melting curve included a gradual increase in the temperature up to 98 ◦C and measuring the fluorescence of the melting amplicon.

Table 5: Primer design for genes analyzed by real-time PCR

Gene	Primer sequences	Annealing temperature (^{0}c)	Accession No	Product size (bp)
IL-6	F: AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT	58	395337	106
Muc2	F: CTGTTGTGGATGGGCGGATTG R:CCAAACTTGCTGTCCAGCTCC	60	XM_032444897	157
IL-4	F: GCTCTCAGTGCCGCTGATG R: GGAAACCTCTCCCTGGATGTC	58	416330	201
iNOS	F: CCTGGAGGTCCTGGAAGAGT R: CCTGGGTTTCAGAAGTGGC	60	NM_204961.1	82
Sod1	F: TGTCCTTTCACTGCTTTCCAT R: TTCCATTGCTGTGTTTGAGGT	58	NM_001167719.1	84
Lxra	F: TGTCCAGAAATGTTCCCAGTGC R: CCTTTGTTCTTATTGGCATCTGTG	62	NM_001079478.1	138
Reference gene				
β actin	F: GAGAAATTGTGCGTGACATCA R: CCTGAACCTCTCATTGCCA	60	L08165	150

F forward primer, R reverse primer, MUC mucin, IL-4 interleukin 4, IL-6 interleukin 6, iNOS nitric oxide synthase, sod1 Superoxide Dismutase, lxra Liver X Receptor and β actin Beta-actin. According to Wang *et al.* (2017) and Zhang *et al.* (2019).

Relative quantification of gene expression:

Using a geometric mean of the two reference genes (β-actin), the expression levels of the target genes (Ct—cycle threshold) were normalized. In order to compute ΔCt, the target genes' Ct was subtracted from the reference genes' Ct (Ct target — Ct reference). Using the Pfaffl method, relative mRNA expression levels were calculated and normalized in relation to the expression level of the housekeeping gene (β-actin).

Histopathological examination:

Autopsy samples were taken from the liver of chicks in different groups and fixed in 10% formol saline for twenty four hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin &eosin stain for examination through the light electric microscope according to Banchroft *et al*. (1996).

Statistical analysis:

Data were analyzed using SAS, 2004 software (SAS, 2004) by using one way ANOVA strains single factor). Tests of significance for the differences between means were carried out according to Duncan (1955).

Model:

$\mathbf{X_{jk} = \mu + S_j + e_{jk}}$ Where: X_{jk} = the k^{th} observation, μ = overall mean, S_j = effect of the j^{th} treatment and e_{jk} = the experimental error.

Results and discussion:

Table 6: Effect of Azolla and probiotics on the intestinal microbiota in broilers chicks of different experimental groups as affected by studied factors

Groups	Parameters					
	Pathogenic bacteria		Beneficial bacteria			
	Total. clost	E. coli	LAB	*B. sub*	Ent.	pH
T1 (Control)	$5.33^{a} \pm 0.06$	$5.62^{abc} \pm 0.15$	$5.79^{e} \pm 0.09$	$5.73^{f} \pm 0.23$	$5.49^{c} \pm 0.2$	$6.8^{e} \pm 0.04$
T2 (5% AZ)	$2.76^{b} \pm 0.11$	$5.84^{a} \pm 0.06$	$6.56^{cd} \pm 0.13$	$6.61^{bcd} \pm 0.13$	$5.31^{c} \pm 0.21$	$7.26^{ab} \pm 0.06$
T3 (10% AZ)	$2.69^{c} \pm 0.14$	$5.23^{de} \pm 0.15$	$6.85^{bc} \pm 0.04$	$6.87^{bc} \pm 0.04$	$5.35^{c} \pm 0.06$	$7.18^{bcd} \pm 0.04$
T4 (15% AZ)	$3.62^{c} \pm 0.13$	$5.75^{ab} \pm 0.12$	$6.62^{cd} \pm 0.05$	$6.51^{cd} \pm 0.1$	$5.45^{c} \pm 0.07$	$7.31^{a} \pm 0.02$
T5 (20% AZ)	$3.5^{b} \pm 0.09$	$5.43^{bcd} \pm 0.1$	$6.55^{cd} \pm 0.2$	$6.24^{de} \pm 0.18$	$5.06^{c} \pm 0.06$	$7.15^{cd} \pm 0.03$
T6 Ent	$3.52^{b} \pm 0.14$	$4.96^{e} \pm 0.07$	$7.58^{a} \pm 0.19$	$7.59^{a} \pm 0.14$	$8.12^{a} \pm 0.05$	$6.28^{g} \pm 0.04$
T7 (5% AZ+ Ent)	$3.42^{b} \pm 0.19$	$5.3^{cde} \pm 0$	$7.26^{ab} \pm 0.23$	$6.82^{ab} \pm 0.1$	$7.8^{ab} \pm 0.07$	$6.49^{f} \pm 0.04$
T8(10% AZ+ Ent)	$3.26^{b} \pm 0.16$	$5.46^{bcd} \pm 0.07$	$6.77^{cd} \pm 0.03$	$6.93^{b} \pm 0.02$	$7.41^{b} \pm 0.09$	$7.08^{d} \pm 0.01$
T9 (15% AZ+ Ent)	$3.33^{b} \pm 0.11$	$5.07^{e} \pm 0.13$	$6.32^{e} \pm 0.19$	$6.87^{bc} \pm 0.08$	$7.77^{ab} \pm 0.41$	$7.28^{ab} \pm 0.02$
T10 (20% AZ + Ent)	$3.22^{b} \pm 0.17$	$5.42^{cde} \pm 0.12$	$5.52^{bcd} \pm 0.09$	$6.01^{ef} \pm 0.03$	$7.84^{ab} \pm 0.33$	$7.19^{bc} \pm 0.01$
PR > F	0.02	0.18	0.34	0.84	<.0001	<.0001

Where: T1: Control group; T2: 5% Azolla; T3: 10% Azolla; T4: 15% Azolla; T5: 20% Azolla; T6: Enterococci; T7; 5% Azolla +Enterococci; T8: 10% Azolla + Enterococci; T9: 15% Azolla + Enterococci; andT10: 20% Azolla + Enterococci. $^{a,b,\ c}$ Means with different superscript in the same column are significantly different at ($P<0.05$). Data are expressed as Mean ± S.E.M for 4 chicken /group.

Obtained data in Table 6 show the effect of azolla and probiotics on cecal in pathogenic bacteria. it was found that in total clostridia were significantly decreased clostridia colonization in caecum of chicks at 35 days of age compared to the control group. Data in E.coli indictor had significant effect to decrease Escherichia coli count in caecal content when compared to control. The results in Table 6 showed the effect of azolla and probiotics on cecal for beneficial bacteria of lactic acid, bacillus subtilis and Enterocooi that treated group with the (T6) Ent and (T7) 5% AZ+ Ent increased signicantly increased compared with control group. Obtained data showed in P_H treated group with T6 decreased caecal pH at 35 days of age compared with control group.

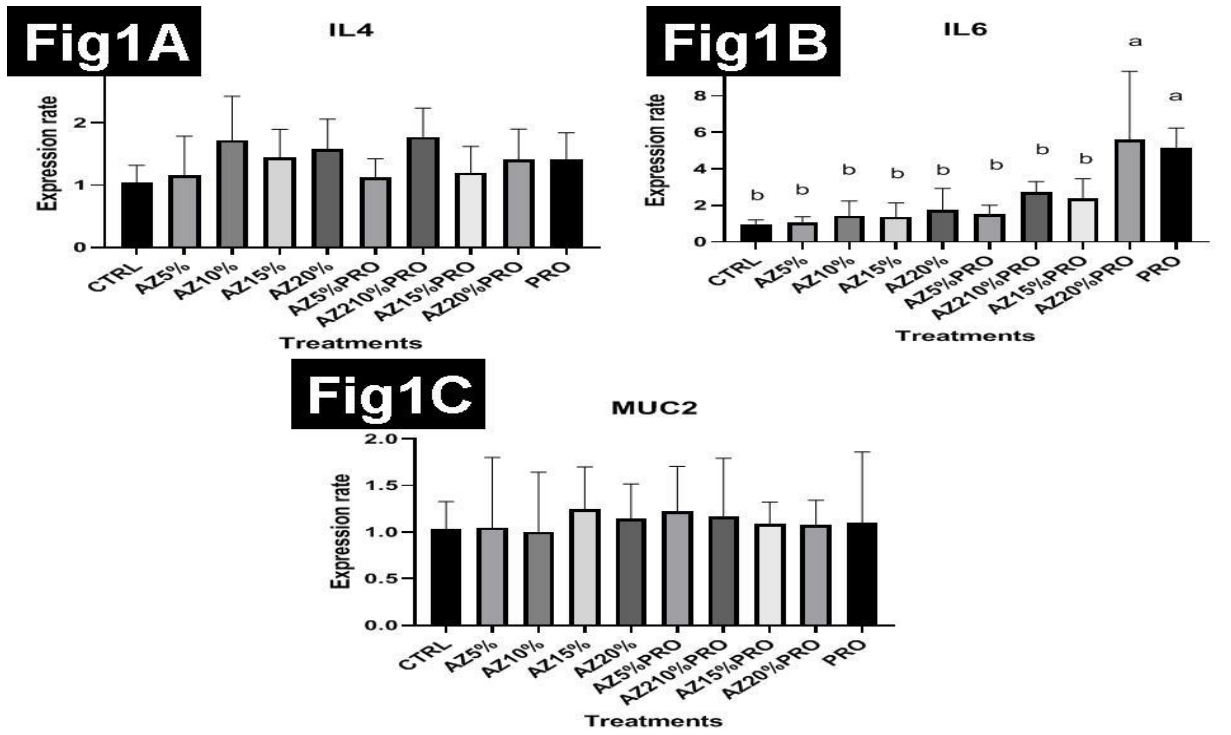


Figure 1: Effect of Azolla and probiotics on genetic immune response markers (IL-4, IL-6 and MUC-2) in broilers chicks' intestinal genes of different experimental groups after 35 days of treatments

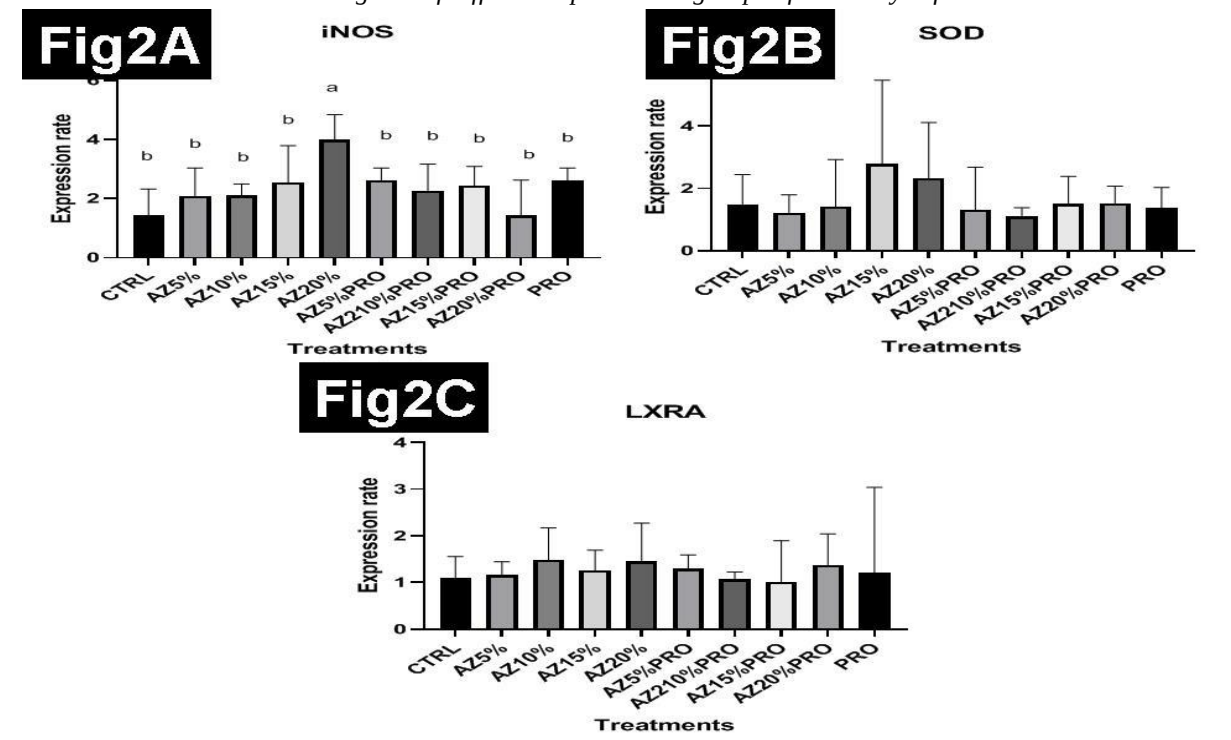
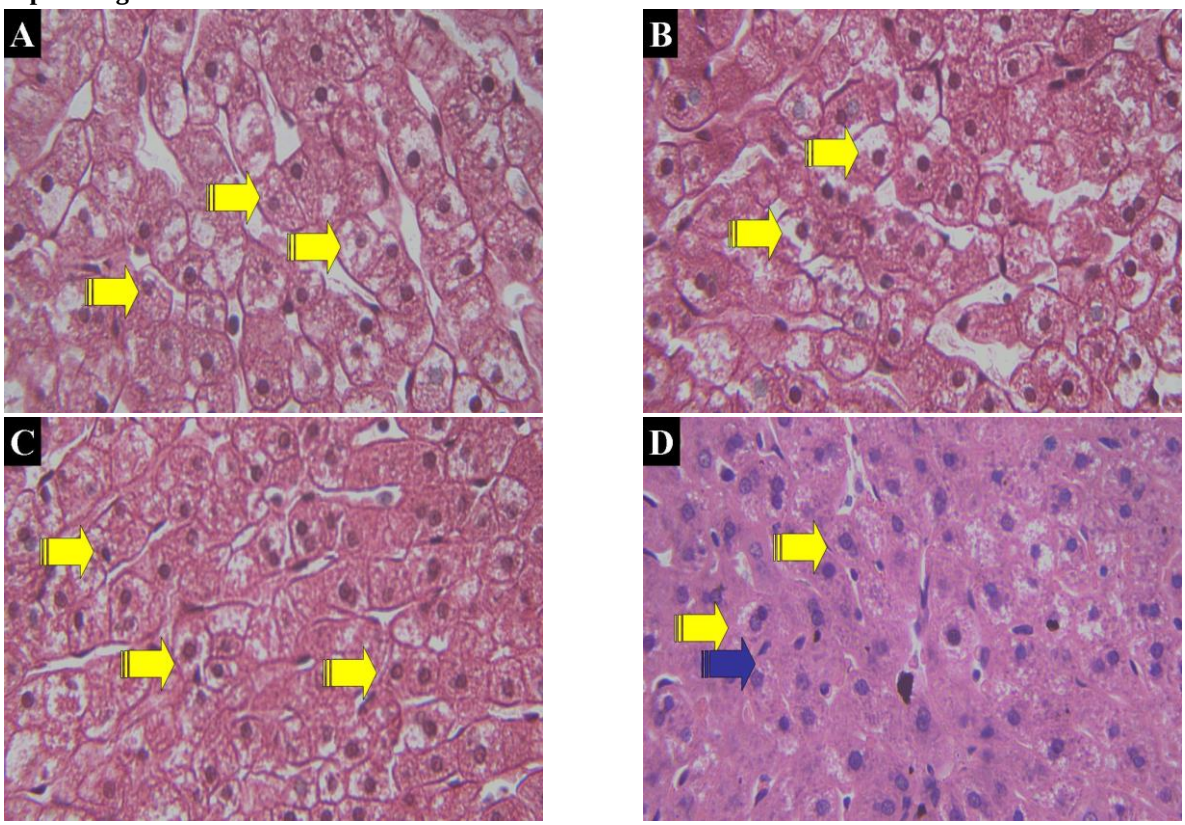


Figure 2: Effect of Azolla and probiotics on antioxidant markers (SOD, INOS, and LXRA) in broilers chicks' liver of different experimental groups after 35 days

Obtained data in Fig. 1 showed that the effect of azolla and probiotics on IL6 in broilers chicks' intestinal was investigating increase for 20% azolla+ pro group in comparing with other groups (Fig 1B) which increase immunogenicity and established the limitation for addition. In contrast, other treated group did not show any significant variation for IL6 which in deed the absent restriction for probiotic or azolla till 15% addition. In the same manner the other immunity parameters IL4 (Fig 1A) and MUC2 (Fig 1C) did not show any significant alteration or variation due to different treatment in comparing with control group.

Obtained data in Fig. 2 showed that the effect of azolla and probiotics on INOS(Fig.2A) in broilers chicks' liver was markedly increase for 20% azolla group in comparing with other group which increase immunogenesity and established the limitation for addition. In contrast, other treated group did not show any significant variation for INOS which in deed the absent restriction for probiotic or azolla till 15% addition. In the same manner the other antioxidant parameters SOD (Fig, 2B), and LXR-α (Fig.2C) did not show any significant alteration or variation due to different treatment in comparing with control group.

Histopathological examination:



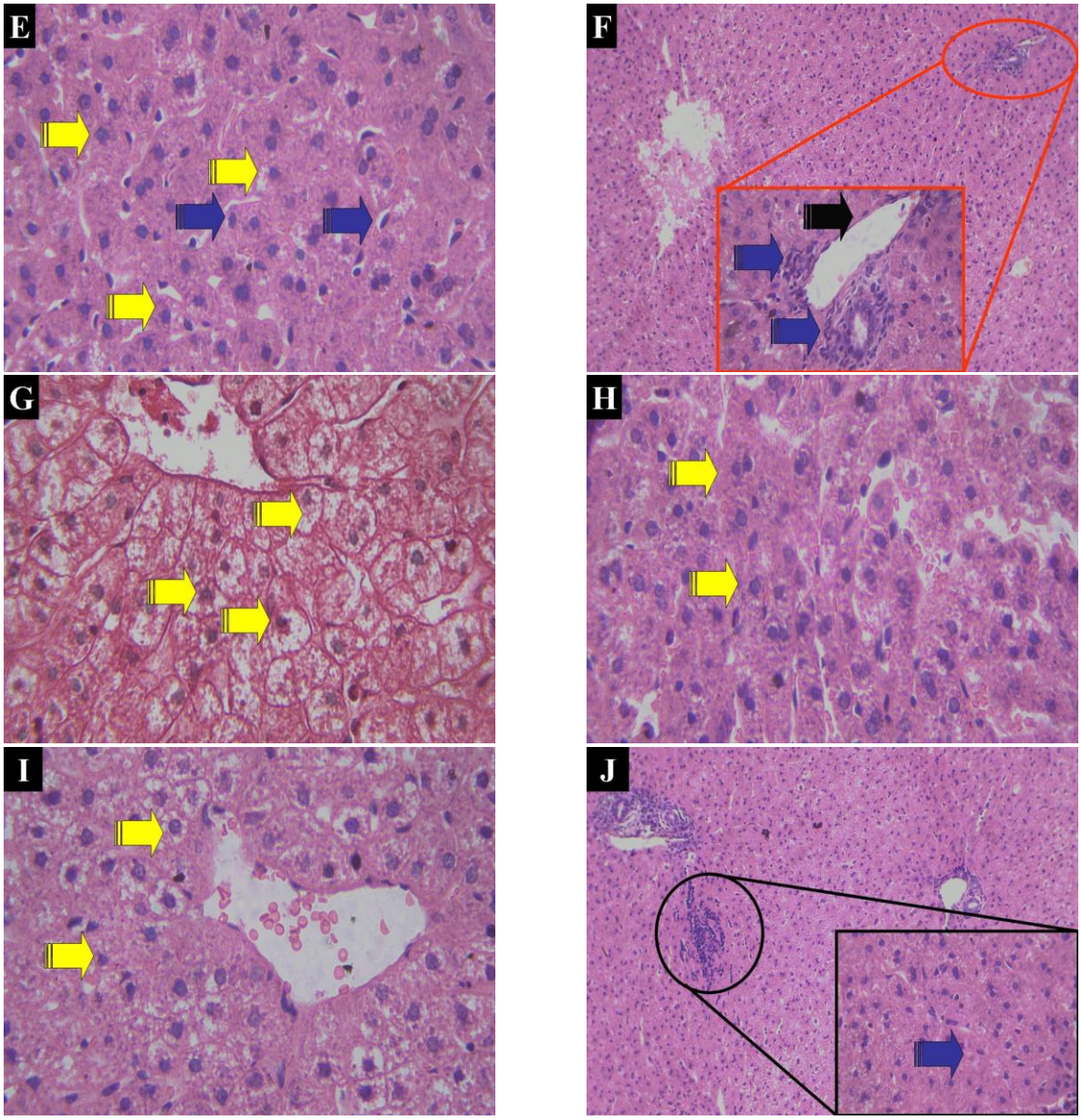


Figure 3: Presented data in figure 3 showed that the microscopically, liver of chicks from control group and other treated groups from A, B, C, D, G, H, I (Control, AZ 5%, Az10, AZ15, EntAZ5, EntAZ10, and EntAZ15, respectively) revealed the normal histological structure of hepatic lobule yellow arrow. Also liver from Fig.3 E, F, J (AZ 20%, Ent, and Ent AZ 20%, respectively) showed mild buffer cell blue arrow and mild lymphocytic infiltration black arrow.

This study was conducted to investigate the effect of feeding different levels of Azolla and probiotic on cecal in intestinal microbiota, gene expression and immune competence traits of Indian River (IR) chicks. Azolla, a type of aquatic fern, has been investigated for its potential antibiofilm activity. Communities of bacteria that stick to surfaces form biofilms and can cause various problems, including persistent infections and resistance to antibiotics. Research studies have explored the ant biofilm properties of Azolla extracts against different bacteria. These studies have indicated that Azolla extracts can inhibit or disrupt biofilm formation by interfering with bacterial adhesion, biofilm matrix formation, or bacterial communication processes (Luzala *et al.*, 2022). Molina Bertrán *et al.*, (2022)

found that Azolla extracts reduced biofilm formation and disrupted pre-formed biofilms of Pseudomonas aeruginosa. it's important to note that the specific mechanisms of action underlying the antibiofilm activity of Azolla extracts are not yet fully understood and require further investigation. Additionally, the effectiveness of Azolla extracts can vary depending on the extraction method, concentration, and specific bacterial strains tested. Organic and aqueous extracts of some Azolla species have shown bioactivity against bacteria and fungi. The heteropolysaccharide produced by the marine bacterium Glutamicibacter nicotianae BPM30, which was isolated from Azolla leaf cavity, demonstrated potential antibiofilm activity (Ravi *et al.,* 2020). In addition the main active polysaccharide of azolla is rhamnose, a naturally occurring sugar molecule, has been studied for its potential antibiofilm activity. Biofilms are complex communities of microorganisms that adhere to surfaces and can cause various problems, including chronic infections and increased antibiotic resistance (Bala Subramaniyan *et al.*, 2019). Research studies have investigated the effects of rhamnose on biofilm formation and its ability to disrupt pre-formed biofilms. These studies have indicated that rhamnose can exhibit antibiofilm properties through various mechanisms (Sen *et al.,* 2020). It was found that rhamnose significantly reduced biofilm formation and disrupted established biofilms of Staphylococcus aureus (Neopane *et al.,* 2018). The study suggested that rhamnose might interfere with the initial attachment of bacteria to surfaces, thus inhibiting biofilm formationIt's important to note that the effectiveness of rhamnose as an antibiofilm agent can vary depending on factors such as concentration, bacterial strains, and experimental conditions. Our results in Table 6 indicted that effect of treatments was highly significant ($P \leq 0.001$) on pathogenic bacteria colonization, beneficial microflora and caecal pH at 35 days. These results are in agreement with Katoch, *et al.* (2021) who stated that the addition of probiotic and Azolla had a significant effect on pathogenic colonization in the caecum of chicks (Qiu *et al.,* 2021). Means of total clostridia count in Table 6 cleared that all treatments were significantly decreased clostridia colonization in caecum of chicks at 35days compared to the control group (treatment No. 1, mean= 10^4 cfu). Obtained are results in agreement with those found by Bostami *et al.*(2016) who reported that addition of Enterococcus faecalis and Azolla inhibit clostridia growth in chicks, while studied the effect of Azolla and Enterococcus faecalis in the drinking water on the clostridia colonization of young broiler chickens. They found that chicks given Enterococcus faecalis had significantly less clostridium count recovered from their caeca than the controls and had a significant increase in the Lactobacillus acidophilus, Bacillus subtilis and Enterococcus faecalis count of their caecal contents (Zhang *et al.,* 2005 and Kemgang *et al.,* 2014). While, there were not significant effect of Azolla on Escherichia coli count. However the addition of Enterococcus faecalis in drinking water had significant effect to decrease Escherichia coli count in caecal content when compared to control (treatment No. 1) (Mookiah *et al.,* 2014). Also, results in Table 6 showed that group treated with Enterococcus faecalis in drinking water and group treated with Enterococcus faecalis with Azolla 5% significantly decreased caecal pH at 35days of age compared with control group. This result could be attributed to the effect of both Enterococcus faecalis which increased lactic acid concentrations of their caecal contents, and consequently directly decreased their caecal pH values (Kim *et al.,* 2016). The same results were obtained by (Abdelatty *et al.*,2021), who stated that the addition of probiotic had a significant effect on caecal pH, while (Ahmed *et al.*,2014) showed that caecal pH did not differ in chick group treated with probiotic from that of the control group (Sadeghi *et al.*,2015). Pathogen identification and destruction is the immune system's main duty. Administering probiotics that boost the local immune system may help to improve this (Fuller *et al.,* 1989), the total tract apparent retention increases when gut microbes are present. TTAR, also referred to as pattern recognition receptors, may stimulate the production of various pro inflammatory cytokines (like IL-6) and antimicrobial peptides (like defensins), which are direct effector molecules of the innate immune response (Ganz ,2003 and Shini ,2010). The capacity of probiotics to block the effects of pro inflammatory cytokines has a significant impact on barrier function. Naïve and T-helper 2 cells both release IL-4 in response to antigenic stimulation; upon activation by antigen recognition, which binds to its receptor, T cells that recognize the antigen begin to proliferate. (Choi and Lillehoj, 2000). T cells and macrophages both generate chicken IL-6, which has anti-inflammatory and pro-inflammatory properties and promotes the synthesis of acute phase proteins (Kaiser *et al.,* 2000; Lynagh *et al.,* 2000; Wigley and Kaiser, 2003). The results presented in Figure 1 shows the effect of Azolla and probiotic supplementation on the gene expression of immunity-related genes in broiler chicks. The mean normalized expression of interleukin 6 (IL6), interleukin 4

(IL4), and mucin 2 (MUC2) was measured in the cecal tissue of different experimental groups of broiler chicks. The results showed that there were no significant differences in the expression of IL4 and MUC2 between the supplemented diet groups and the control group. This suggests that the supplementation of Azolla and probiotics did not have a significant impact on the expression of these immunity genes in the cecal tissue of the broiler chicks (Amer *et al.*, 2023).However, the results showed that the supplementation of Azolla at 20% in combination with probiotics significantly increased the expression of IL6 compared to the control group. This indicates that the combined supplementation of Azolla and probiotics may have a positive effect on the expression of pro-inflammatory genes in the cecal tissue of the broiler chicks (Pender *et al.*, 2017 and Abdelatty *et al.*, 2021). The results of Figure 2 suggest that the supplementation of Azolla and probiotics in the diets of broiler chicks has a varying effect on the gene expression of antioxidant-related genes, such as superoxide dismutase 1 (SOD1), inducible nitric oxide (INOS), and Liver X receptor alpha (LXRA), in the cecal tissue. The mean normalized expression of these genes was measured and compared between different treatment groups and the control group according to Magouz *et al.* (2020). The results showed that there were no significant differences in the expression of SOD1 and LXRA between the supplemented diet groups and the control group. This suggests that the supplementation of Azolla and probiotics did not have a significant impact on the expression of these antioxidant genes in the cecal tissue of the broiler chicks. However, the results showed that the supplementation of Azolla at 20% significantly increased the expression of INOS compared to the control group. Demeure *et al.* (2009) indicates that Azolla supplementation may have a positive effect on the expression of pro-inflammatory genes in the cecal tissue of the broiler chicks.

Conclusion

Using Azolla as replacement level of 5% of soy protein and without probiotic *(Enterococcus faecails)* improve beneficial microflora and reduce pathogens in caecum. And the results suggest that the supplementation of Azolla and probiotics in the diets of broiler chicks has a varying effect on the gene expression of immunity-related genes and antioxidant-related genes in the cecal tissue. Further Research is necessary for understanding the underlying mechanisms of these effects and to determine the optimal levels of supplementation for optimal health and growth of broiler chickens.

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