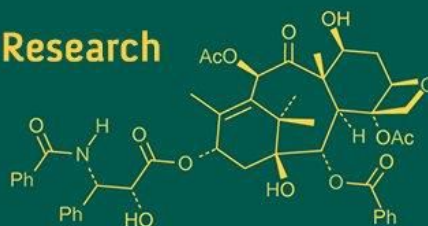
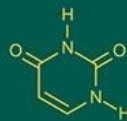
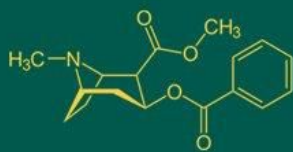


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S Singour

Ph.D. Scholar, Department of Animal Nutrition, College of Veterinary Science & A.H. (Jabalpur), Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

S Nayak

Professor and Head, Department of Animal Nutrition, College of Veterinary Science & A.H., Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

A Khare

Assistant Professor, Department of Animal Nutrition, College of Veterinary Science & A.H. (Jabalpur), Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

AP Singh

I/C Dean and Professor, Department of Animal Genetics and Breeding, College of Veterinary Science & A.H. (Rewa), Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

G Goyal

Assistant Professor, Department of Poultry Science, College of Veterinary Science & A.H. (Jabalpur), Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

RK Barhaiya

Assistant Professor, Department of Veterinary Anatomy, College of Veterinary Science & A.H. (Jabalpur), Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

K Shrivastav

Assistant Professor, Department of Animal Biotechnology Centre, College of Veterinary Science & A.H. (Jabalpur), Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

YA Rather

Ph. D. (Scholar), Department of Poultry Science, College of Veterinary Science & A.H. (Jabalpur), Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

R Chaurasia

Ph. D. (Scholar), Department of Veterinary Medicine, College of Veterinary Science & A.H. (Mhow), Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

Corresponding Author:**R Chaurasia**

Ph. D. (Scholar), Department of Veterinary Medicine, College of Veterinary Science & A.H. (Mhow), Nanaji Deshmukh Veterinary Science University, Jabalpur, Madhya Pradesh, India

Effect of dietary supplementation of *Bacillus subtilis* & *Bacillus clausii* based probiotics alone or in combination on expression of (tlr4 & occludin) gene in Jabalpur colour birds

S Singour, S Nayak, A Khare, AP Singh, G Goyal, RK Barhaiya, K Shrivastav, YA Rather and R Chaurasia

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Abstract

The study was conducted to investigate the effect of dietary supplementation of *Bacillus subtilis* & *Bacillus clausii* based probiotics alone or in combination on expression of (TLR4 & occludin) gene in Jabalpur colour birds. A total of two hundred forty, 30 weeks age laying hens Jabalpur colour bird were selected from the poultry farm, adhartal Jabalpur. These layers were randomly distributed to five replicates of six layers each and allotted to 8 dietary treatments. The standard layer diet (T0) was formulated as per ICAR (2013) specification. T₁: Control diet + antibiotic (avilamycin@100 g/ ton of feed) T₂: Control diet + *Bacillus subtilis* (2× 10⁹ cfu/g) @250 g /ton of feed) T₃: Control diet + *Bacillus subtilis* (2× 10⁹ cfu/g) @500 g /ton of feed) T₄: Control diet + *Bacillus clausii* (2× 10⁹ cfu/g) @250g /ton of feed) T₅: Control diet + *Bacillus clausii* (2× 10⁹ cfu/g) @500 g /ton of feed) T₆: Control diet + *Bacillus subtilis* (125 g) + *Bacillus clausii* (125g) (2× 10⁹ cfu/g) @250 g/ ton of feed) T₇: Control diet + *Bacillus subtilis* (250 g) + *Bacillus clausii* (250g) (2× 10⁹ cfu/g) @500 g/ ton of feed) respectively for 12 weeks of experimental period. it was concluded that supplementation of *Bacillus subtilis* and *Bacillus clausii* (@125 g/ton of feed each) based probiotics had upregulation of TLR-4 and occludin gene. real-time expression profiling of the Occludin (OCN) gene revealed differential regulation among the treatment groups compared with the control (T0) (at different levels) after 90 days trial. The highest upregulation of OCN mRNA expression was observed in group T₅, with a fold change value of 2.53, followed by T₂ (1.87). Groups T₁ (0.84), T₆ (0.47), and T₇ (0.62) showed downregulation relative to the control, while group T₃ maintained expression similar to T0 (1.00). Notably, the lowest expression level was recorded in T₄, with a fold change value of 0.05. Overall, the supplementation strategies in groups T₂ and T₅ resulted in marked upregulation of OCN expression, with T₅ exhibiting the maximum fold increase. In contrast, groups T₁, T₄, T₆, and T₇ showed downregulation of OCN expression compared to the control. In the present study, expression profiling of the Toll-like receptor (TLR) gene showed variable regulation across the treatment groups when compared with the control or basal diet (T0). The highest upregulation was observed in T₂ (2.90-fold) and T₅ (2.86-fold), followed by T₄ (1.27-fold) and T₆ (1.13-fold). Groups T₁ (0.95) and T₇ (0.94) exhibited slight downregulation relative to the control, whereas T₃ showed expression comparable to T0 (1.00). Overall, the supplementation strategies in groups T₂, T₅, T₄ and T₆ elicited the strongest upregulation of TLR gene expression, while groups T₁ and T₇ showed marginal downregulation.

Keywords: *Bacillus subtilis* & *Bacillus clausii*, Probiotics, Jabalpur Colour, Occludin and TLR genes

1. Introduction

Currently, the poultry population in India is 851.81 million (20th Livestock Census) and egg production is around 129.60 billion during 2021-22. The per capita availability during 2021-22 is around 95 eggs per annum. The Egg production has shown positive growth as 6.19% during 2021-22.

For enhancing the productivity of laying hens and lower the incidence of disease commercially, a huge number of antibiotic growth promoters (AGPs) are given. Overuse of antibiotics at doses below therapeutic levels resulted in the development of superbugs, germs that are resistant to antibiotics and persists in both human and animal food chains (Xiang *et al.*, 2019) [8]. Consequently, the European Union has restricted the use of AGPs in feed since 2006. Therefore; alternatives to antibiotics are urgently needed.

Probiotics of various commercial preparations are being considered worldwide for poultry as performance enhancers and suitable alternatives to antibiotics. The probiotics are considered as “direct-fed microbial” and they affect the host positively by balancing the intestinal microbial populations. The efficacy of probiotics depends on number of factors of which the composition of microbial species, the dose of each preparation, methods of administration, diet composition, length of feeding, the genotype of the birds and the environment to which birds are exposed found most remarkable (Jha *et al.*, 2020) [2]. These factors make it difficult to predict the exact effects of probiotics (Saksrithai and King, 2019) [7] unless they are individually tested for their efficacy. Among these contributing factors, the composition of probiotics organisms in a preparation that makes probiotics either single- or multi-strain and/or their doses plays a vital role.

It is well recognized that probiotics organisms are beneficial for the host animals (Mazanko *et al.*, 2017) [5] and the most commonly used probiotics organisms are *Lactobacillus spp.*, *Bacillus spp.*, *Enterococcus spp.*, *Streptococcus thermophilus*, *Bifidobacterium spp.*, *Escherichia coli* and fungal species.

Of these, *Bacillus spp.* is gaining acceptance in use due to its spore-forming ability and among the fungal species, *Saccharomyces cerevisiae* is the most widely used one. Spore-forming probiotics, (SFPs) especially *Bacillus* species, (like *Bacillus Subtilis* and *Bacillus clausii*) have promising approach over other probiotics. Due to their encapsulation sporulation ability SFPs can reach the specific part of the gastrointestinal (GI) tract easily which is associated with their survival and colonization in the digestive tract (Khalid *et al.*, 2022) [4].

Furthermore, *Bacillus* probiotics biofilm included biochemical effects like antimicrobial, enzymatic activity, modulating immune system, helping to preserve GI tract and prevent other infection and spore demonstrate tremendous tolerance survivability in extreme temperature, pH, dehydration and malnutrition (Pan *et al.*, 2022) [6].

SFPs Probiotics produce several benefits on layer health by modulating the gut health and intestinal barrier function, increase egg production, egg quality (yolk cholesterol, improve shell thickness egg weight) quantities and qualitative composition of micro biota, improve feed to egg ratio, play important role in immune modulation, pro-inflammatory cytokine production and macrophages activation without cytotoxicity. Many investigators have pointed out that probiotics could reduce the cholesterol content of egg yolk and serum (Fathi *et al.*, 2018) [1].

2. Material and Methods

2.1 Location and Place of work

The experiment was conducted in the Department of Animal Nutrition and feeding trial was conducted at Poultry farm, Adhartal, Veterinary College, Jabalpur (M.P.). The comprehensive programme of work was as follows:

2.2 Experiment

The study was planned to investigate the effects of dietary supplementation of different designer diets on performance, serum and yolk cholesterol concentration, immune response and economics of enriched egg production.

2.3 Housing

The experimental layer of 30 weeks age were reared in the cage layer house. The house were cleaned, washed and disinfected by blow lamping and complete house was fumigated using formaldehyde and potassium permanganate four days prior to start of the experiment. Feeders and waterers were carefully cleaned with detergent.

2.4 Duration of Experiment

Experiment was conducted for a period of twelve weeks. It was started on 25th October 2021 and terminated on 17th January 2022.

2.5 Experimental diet

2.5.1 Feed

The dietary ingredients like maize, soybean meal and rice polish, other supplements was procured from the Poultry farm feed store. These ingredients were analyzed for proximate principles and energy before formulation of diets. The experimental diets were formulated as per ICAR (2013) layers of age > 30 weeks and weight 1400 g specification.

Table 1: Proximate composition of feed ingredients (% DM basis)

Constituents (%)	Maize	Soybean meal	Rice Polish
Moisture*	11.82	11.73	9.20
Crude protein*	8.80	46.00	11.50
Ether extract*	3.20	1.34	11.50
Crude fibre*	1.90	7.50	10.80
NFE	69.16	27.11	45.20
Ash*	5.12	6.32	11.80
ME (Kcal/kg)	3210	2180	2820

*Value analyzed in the laboratory

2.5.1.1 Dietary treatments

Six isonitrogenous and isocaloric dietary treatments were as follows:

Two hundred and forty layers was randomly distributed to 5 replicates of 6 layers each and allotted to following 8 dietary treatments:

- **T0:** Control layer mash (corn, soya, DORB diet) without enrichment
- **T1:** Control diet + antibiotic (avilamycin@100 g/ ton of feed)
- **T2:** Control diet + *Bacillus subtilis* (2×10^9 cfu/g) @250 g /ton of feed)
- **T3:** Control diet + *Bacillus subtilis* (2×10^9 cfu/g) @500 g /ton of feed)
- **T4:** Control diet + *Bacillus clausii* (2×10^9 cfu/g) @250 g /ton of feed)
- **T5:** Control diet + *Bacillus clausii* (2×10^9 cfu/g) @500 g /ton of feed)
- **T6:** Control diet + *Bacillus subtilis* (125g) + *Bacillus clausii* (125g) (2×10^9 cfu/g) @250 g/ ton of feed)
- **T7:** Control diet + *Bacillus subtilis* (250g) + *Bacillus clausii* (250g) (2×10^9 cfu/g) @500 g/ ton of feed)

2.5.1.2 Computation of Ration

The feed ingredients were procured from the market and analyzed for proximate composition before formulation of diets. The experimental diets were formulated as per layer feed (ICAR, 2013), (16.5% CP and 2600 kcal ME/kg diet). The analyzed protein and ME values of feed ingredients were used for computation of rations. Composition of experimental layer diets used in the study is given in table 02.

Table 2: Ingredient composition of layer experimental diets

Feed ingredient	Experimental diets (%)					
	T0	T1	T2	T3	T4	T5
Maize (kg)	450	450	450	450	450	450
Rice Polish (kg)	250	250	250	250	250	250
Soyaben meal (kg)	221	221	221	221	221	221
Shell grit (kg)	40	40	40	40	40	40
Calcite/LSP(kg)	25	25	25	25	25	25
Dicalcium phosphate (kg)	2.5	2.5	2.5	2.5	2.5	2.5
Mineral premix(kg)	2.5	2.5	2.5	2.5	2.5	2.5
Vitamin premix (kg)	2.5	2.5	2.5	2.5	2.5	2.5
Salt (kg)	6.5	6.5	6.5	6.5	6.5	6.5
Total	1000	1000	1000	1000	1000	1000
Nutrient composition calculated (%):						
Crude protein	16.5	16.5	16.5	16.5	16.5	16.5
Ca	3.26	3.26	3.26	3.26	3.26	3.26
Total P	0.44	0.45	0.44	0.44	0.44	0.45
Energy (Kcal ME/kg diet)	2602	2599	2602	2602	2602	2599

2.5.2 Experimental birds

A total of two hundred forty, 30 weeks age laying hens (Jabalpur colour bird) were selected from the Poultry farm, Adhartal of Veterinary College Jabalpur. These layers were randomly distributed to five replicates of six layers each and were allotted to 8 dietary treatments.

2.5.3 Experimental design

The design of the experiment was completely randomized design. All the layers of 30 weeks age were individually weighed at the start of the experiment and 240 birds of identical weight were selected. The birds were randomly assigned to various groups so that weight of the layers in any two groups did not differ significantly. Overall, there were 8 dietary treatments. Each treatment consisted of five replicates of six layers each.

2.5.4 Feeding and Watering

The feed was offered *ad libitum* per requirement in linear feeders. All-mash system of feeding was practiced during the experiment. Fresh and clean drinking water was made available to birds all the time. Thus, in the entire study uniform condition of housing, feeding and watering was maintained for all the groups of the experiment.

2.6 Methods

2.6.1 Measurements and Observations

Expression of profiling gene related to immune response (TLR4) and intestinal integrity (Occludin) in layer

To evaluate the expression of target gene related to immune response (TLR4) and intestinal integrity (Occludin) total RNA was isolated from the collected intestine samples of all treatment groups at day 90 using Trizol reagent and mRNA was reversely transcribed into cDNA using TAKARA PrimeScript™ kit. Then quantitative real-time polymerase chain reaction (qRT-PCR) was performed using designed primers (Table 00). Gene expression levels were analyzed using the $\Delta\Delta C_t$ method after normalization against the pooled expression levels of housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Faot *et al.*, 2017 and Xiang *et al.*, 2019)^[8].

Isolation of RNA from chicken intestine

About 500 mg intestine tissue sample was taken in a DEPC treated micro-centrifuge tube and was submerged in trizol reagent.

- Then samples were flash frozen and stored at -80 °C prior to RNA isolation. The samples were thawed and brought to room temperature for further processing, the samples were stored at -20 °C for at least 1 hour.
- Thawed intestine samples were weighed and a sample of 100mg was utilized for RNA isolation. Sample was homogenized by help of liquid nitrogen and then transferred to a fresh 1.5 ml micro-centrifuge tube containing 1 ml of trizol reagent and mixed by gentle inversion for 5 min at room temperature.
- Then 0.2 ml of chilled chloroform was added to the tube and mixed by gentle vortexed and incubated at room temperature for 2-3 min.
- The samples were then centrifuged at 12,000 rpm for 15 minutes at 4 °C which formed two different layers, the upper aqueous phase was transferred in a 1.5 ml eppendorf tube. Then 0.5 ml chilled isopropyl alcohol was added and incubated at -20 °C for 1 hr.
- The samples were centrifuged for 15 minutes at 12,000 rpm at 4 °C which resulted in formation of visible white pellet at the bottom of the tube.
- Supernatant was discarded, 200 μ l of 75% ethanol was added and centrifuged the sample for 10 minutes at 10,000 rpm at 4 °C.
- Ethanol washing was done 2-3 times and then the supernatant was discarded and pellet was air dried in laminar air flow chamber for 10 minutes.
- Air dried pellets were resuspended in 25 μ L of DEPC-treated water and sample was incubated for 20 minutes at 40 °C.
- Sample was quantified using Nanodrop™ 2000/2000c spectrophotometer then stored at -80 °C.

Spectrophotometric quantification of mRNA

To assess the purity and integrity of isolated mRNA, samples were quantified using Nanodrop™ 2000/2000c spectrophotometer. 1 μ L of RNA sample was used for the quantification of RNA. O.D. of sample was measured at 260nm and 280nm wavelengths against nuclease free milliQ water as a blank. Samples with A (260/280) values between 2.0-2.2 were processed further.

cDNA synthesis from RNA

PCR conditions were standardized to amplify the gene sequences using reverse transcriptase polymerase chain reaction (RT-PCR). Gene specific primers were used for the

amplification and annealing temperature was standardized by using cDNA synthesized from RNA samples. cDNA was synthesized from TAKARA PrimeScript™ first strand

cDNA synthesis kit. The procedure of cDNA synthesis, in brief, is as follows:

Table 3: Components of reaction mixture for cDNA synthesis for first reaction

S.No.	Reagent (First Reaction)	Quantity
1	Primer oligo DT (50 µM)	1.0 µL
2	dNTP mixture (10 mM each)	1.0 µL
3	Template RNA (110 ng/µl)	1.0 µL
4	RNase free dH ₂ O	7.0 µL
	Total volume	10 µL

- The following mixture was prepared in a microtube (Table 03)
- The above mixture was incubated for 5 min. at 65 °C, then cooled immediately on ice.
- Second reaction mixture was prepared with a total volume of 20 µL (Table 05).

Table 4: Components of reaction mixture for cDNA synthesis for second reaction

S.No.	Reagent (Second Reaction)	Quantity
1	Template RNA Primer Mixture (from step 02)	10 µL
2	5X Primer Script Buffer	4.0 µL
3	RNase Inhibitor (40 U/µl)	0.5 µL (20 U)
4	Prime Script RTase (200 U/µl)	1.0 µL (200 U)
5	RNase Free dH ₂ O	4.5 µL
	Total volume	20 µL

- The above reaction mix was mixed gently.
- The reaction mixture was incubated at 42°C for 60 min
- Inactivation of enzyme was done by incubating at 70°C for 15 min followed by ice cooling.
- After stopping the reaction in step 6, the cDNA was stored at -20°C for further use. This cDNA was used for expression profiling of target genes.

Standardization of oligonucleotide primers for real time expression profiling

For expression profiling of TLR4 and Occludin, gene specific primers were used as given in Table 06. The primers were diluted first to make the stock solution (100 pmole/µl), which was again diluted to make a working solution of final concentration 10 pmole/µl.

Table 4: Details of primers and their sequence used in RT-PCR

S.No.	Primer Name	Sequence (5'-3')	length	Expected Amplicon size (bp)	Reference
1	MstnChicken_F	TACCCGCTGACAGTGGATTTC	21	153	Li <i>et al.</i> (2021)
2	MstnChicken_R	GCCTCTGGGATTTGCTTGG	19		
3	GAPDHPoultry_F	ACATGGCATCCAAGGAGTGAG	21	144	Li <i>et al.</i> (2021)
4	GAPDHPoultry_R	GGGGAGACAGAAGGGAACAGA	21		

To ascertain the optimum annealing temperature, the primers were standardized using gradient PCR technique. The steps for standardization are as follows -

- Preparation of PCR reaction mix - for preparation of standard PCR reaction mix, different components were thawed carefully on ice pack and were added in quantities as given in table 07. The components were added to make final volume 25 µ, spined briefly to mix them properly and positioned in PCR as per the temperature, for primer standardization (Ref Step 02).

Table 5: Standardized PCR reaction mixture.

S. No.	Reagent	Quantity
1	PCR master mix	12.5 µL
2	Forward primer (10%)	1.0 µL
3	Reverse primer (10%)	1.0 µL
4	Nuclease free water	7.5 µL
5	cDNA template	3.0 µL
	Total volume	25 L

2. Setting up of gradient temperature

Temperature was set in gradient manner from 56.0 °C to 61.0 °C to standardize the annealing temperature of

oligonucleotide primers. The temperature gradient and arrangement of PCR tubes in thermal cycler is given in Table 08. The standardized PCR programme for primer standardization is given in Table 09.

Table 6: Temperature gradient setup for standardization of oligonucleotide primers used for expression profiling.

Temperature (°C)	55.0	56.0	57.0	58.0	59.0	60.0
Well no.	A1	A3	A5	A7	A9	A11
Reaction tubes	M1	M2	M3	M4	M5	M6
	G1	G2	G3	G4	G5	G6

Table 7: PCR programme for standardization of TLR4 and occludin gene

Sr. no.	Step	Temperature	Time
1.	Initial denaturation	98 °C	30 s
2.	Denaturation	98 °C	10 s
3.	Annealing	56 °C - 61 °C	30 s
4.	Extension	72 °C	30 s
Step 2- 4 repeated 35 times			
5.	Final extension	72 °C	2 min
6.	Final hold	4°C	

- The PCR products obtained, were run on 1.2% (w/v) agarose gel with a standard DNA marker, in horizontal gel electrophoresis for size-based separation and visualization of amplicons.
- The gel was photographed under gel documentation system (Ingenius, Syngene) and each product was analyzed for intensity / quality of amplification, to ascertain the optimum annealing temperature.
- The temperature at which clear cut single amplification band of appropriate size was obtained, was noted as standardized annealing temperature for the respective primer.

Quantitative real-time PCR (qRT-PCR)

All real time PCR reactions were performed in triplicate. The RT-qPCR was done using Fast SYBR® Green Master Mix (Applied Biosystems, Life Technologies). The amplification was carried out in 10 µl volume reaction mixture containing 5 µl of 2X Fast SYBR® Green Master Mix, 1 µl (10 pmol/µl) of gene specific forward and reverse primer, 1.5 µl of cDNA template and nuclease free water to make volume (Table 10). Real-Time PCR was carried out in Applied Biosystems™ 7500 Real-Time PCR (Life Technologies, Carlsbad, USA). The thermal profile used for this was as follows: 95 °C for 20 sec then 40 cycles of 95 °C for 3 sec, 60 °C for 01:00 min followed by melt curve analysis which consisted of denaturation at 95 °C for 15 sec., renaturation at 60 °C for 1 min. and denaturation ramp up to 95 °C @ 0.3 °C/6 sec. for 15 sec. (Table 11). Fold changes in target transcript levels were determined by using the method described by Livak and Schmittgen (2001). GAPDH was used as internal house-keeping gene (endogenous control) for assessment of fold change in target genes.

Table 8: Master mix preparation for real-time PCR (1X)

Components	Volume per reaction (µL)
2X Fast SYBR® Green Master Mix	5.0
Forward Primer (10p mole/ µl)	1.0
Reverse Primer (10p mole/ µl)	1.0
cDNA template	1.5
Molecular biology grade water	1.5

Table 9: Standardized PCR programme for expression profiling by real time PCR

S.No.	Step	Temp. (°C)	Duration	Cycle
1	Activation	95.0	20 sec.	Hold
2	Denaturation	95.0	15 sec.	40 cycles
3	Anneal/Extend	60.0	1 min.	
Melt curve analysis				
4	Denaturation	95.0	15 sec.	
5	Renaturation	60.0	1 min.	
6	Denaturation ramp rate	Up to 95°C @ 0.3°C/6 sec. for 15 sec.		

Gene expression analysis

Once the Ct value was collected for each reaction, it was used to generate a relative expression level. In this experiment, there were twelve samples from all the groups. Their relative expression was estimated using the method described by Livak and Schmittgen (2001).

3. Results and Discussion

The objective of this study was to assess the effect of *Bacillus subtilis* and *Bacillus clausii* based probiotics, administered alone or in combination, on the production performance of Jabalpur colour birds. A total of two hundred forty, 30 weeks age laying hens Jabalpur colour bird were randomly distributed to five replicates of six layers each and allotted to 8 dietary treatments. The standard layer diet (T0) was formulated as per ICAR (2013) specification. T₁: Control diet + antibiotic (avilamycin@100 g/ ton of feed) T₂: Control diet + *Bacillus subtilis* (2× 10⁹ cfu/g) @250 g /ton of feed) T₃: Control diet + *Bacillus subtilis* (2× 10⁹ cfu/g) @500 g /ton of feed) T₄: Control diet + *Bacillus clausii* (2× 10⁹ cfu/g) @250g /ton of feed) T₅: Control diet + *Bacillus clausii* (2× 10⁹ cfu/g) @500 g /ton of feed) T₆: Control diet + *Bacillus subtilis* (125g) + *Bacillus clausii* (125g) (2× 10⁹ cfu/g) @250 g/ ton of feed) T₇: Control diet + *Bacillus subtilis* (250g) + *Bacillus clausii* (250g) (2× 10⁹ cfu/g) @500 g/ ton of feed).

Effect of single and multi-strain probiotics supplementation on expression profiling of genes TLR4 and Occludin in Jabalpur colour birds

Expression profiling of TLR4 and Occludin gene

Expression profiling was done by using SYBR green protocol in RT-PCR. In real-time PCR, the amount of cDNA is measured after each cycle via fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of PCR product molecules (amplicons) generated. Data collected in the exponential phase of the reaction yield quantitative information on the starting quantity of the amplification target. By plotting fluorescence against the cycle number, it generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction.

Occludin is a key tight junction protein that plays a crucial role in maintaining tight junction integrity and ensuring optimal barrier function. Supplementation with *B. subtilis* markedly increased occludin mRNA expression levels in laying hens, indicating an enhancement of gut barrier integrity and intestinal defense mechanisms. Toll-like receptors (TLRs), essential pattern-recognition receptors in both mammals and birds, are key mediators of the innate immune response. By recognizing pathogen-associated molecular patterns (PAMPs) of microorganisms, TLRs initiate signaling pathways that activate genes involved in anti- or pro-inflammatory cytokine production, thereby triggering subsequent immune responses (Kawasaki and Kawai, 2014) [3].

This study was designed to investigate the TLR4 and Occludin gene mRNA expression in Jabalpur colour birds supplemented with different level of supplemented probiotics in supplemented probiotics alone *bacillus subtilis* (250 or 500 g/ton feed each) and *bacillus clausii* (250 or 500 g/ton feed each) or in combination *bacillus subtilis* and *bacillus clausii* (@125 or 250 g/ton feed each) with taking housekeeping gene (GAPDH) as reference gene to normalized the data.

Experimental protocol

To evaluate the expression of target gene TLR4 and Occludin, total RNA was isolated from the collected intestine tissue of all treatment groups at 42th week using Trizol reagent and mRNA was reversely transcribed into

cDNA using Takara Primescript™ for further expression profiling study. The expression levels were studied using qRT-PCR SYBR green assay.

The Ct value of each sample was recorded and average Ct value was calculated. Values of GAPDH were used to calculate the relative quantification (fold change) of the gene included in the study. The standardized annealing temperature for oligonucleotide primers was obtained at 55.0 °C. The amplification product, amplification plot and

melt curve for genes are shown in Table 29, Fig. 19 and 20, respectively. The analysis of real time data was done to determine the significant differences between Δ Cts of the analyzed groups. Data obtained was normalized to the reference gene and expressed as fold change. The data was analysed using the delta delta Ct method of Livak and Schmittgen and was expressed as fold change with respect to the control group.

Table 10: Gene expression profiling of Occludin and TLR- 4 gene of Jabalpur colour birds

Group	Occludin	Regulation	TLR - 4	Regulation
T0	1.00	Control	1	Control
T ₁	0.84	Down regulated	0.95	Down regulated
T ₂	1.87	Up regulated	2.9	Up regulated
T ₃	1.0	Equivalent to control	1.0	Equivalent to control
T ₄	0.05	Down regulated	1.27	Up regulated
T ₅	2.53	Up regulated	2.86	Up regulated
T ₆	0.47	Down regulated	1.13	Up regulated
T ₇	0.62	Down regulated	0.94	Down regulated

In the present study, real-time expression profiling of the Occludin (OCN) gene revealed differential regulation among the treatment groups compared with the control (T0) (at different levels) after 90 days trial. The highest upregulation of OCN mRNA expression was observed in group T₅, with a fold change value of 2.53, followed by T₂ (1.87). Groups T₁ (0.84), T₆ (0.47), and T₇ (0.62) showed downregulation relative to the control, while group T₃ maintained expression similar to T0 (1.00). Notably, the lowest expression level was recorded in T₄, with a fold change value of 0.05.

Overall, the supplementation strategies in groups T₂ and T₅ resulted in marked upregulation of OCN expression, with T₅ exhibiting the maximum fold increase. In contrast, groups T₁, T₄, T₆, and T₇ showed downregulation of OCN expression compared to the control.

In the present study, expression profiling of the Toll-like receptor (TLR) gene showed variable regulation across the treatment groups when compared with the control or basal diet (T0). The highest upregulation was observed in T₂ (2.90-fold) and T₅ (2.86-fold), followed by T₄ (1.27-fold) and T₆ (1.13-fold). Groups T₁ (0.95) and T₇ (0.94) exhibited slight downregulation relative to the control, whereas T₃ showed expression comparable to T0 (1.00).

Overall, the supplementation strategies in groups T₂, T₅, T₄ and T₆ elicited the strongest upregulation of TLR gene expression, while groups T₁ and T₇ showed marginal downregulation.

Our study was in concurrence with Zhang *et al.* (2021) [8] conducted a study on supplementation of *Bacillus subtilis* YW1, on the gene expression of laying hens. They resulted that diet supplemented with *B. subtilis* (2.5 10⁹ CFU/kg), displayed a statistically significant upregulation the tight junction protein occludin, compared to basal diet however, there was no statistically significant difference in TLR1, TLR2, TLR4, TLR15, TNFSF15, or LYZ mRNA expression levels were observed. Similarly Zou *et al.*, found that supplementation of *Bacillus subtilis* (0.5 g/kg) increased mRNA expression of tight junction protein occludin ($P < 0.05$), while reduced mRNA expression of lipopolysaccharide-induced TNF factor ($P < 0.01$) in the duodenum. Also in contrast to our study Chen *et al.*, reported that the mRNA expression levels of claudin-1 and

zonula, occludens-1 in the duodenum or jejunum were not affected by BS and MMT supplementation. An interaction effect between BS and MMT supplementation was observed in the mRNA expression level of occludin in the jejunum. The BS supplementation reduced the mRNA expression levels of TLR4 and IL-1b in the duodenum.

4. Conclusions

It was concluded that supplementation of *Bacillus subtilis* and *Bacillus clausii* (@125 g/ton of feed each) based probiotics had upregulation of TLR-4 and occludin gene.

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References

- Fathi M, Homidan I, Dokhail A, Ebeid T, Abou-Emera O, Alsagan A. Effects of dietary probiotic (*Bacillus subtilis*) supplementation on productive performance, immune response and egg quality characteristics in laying hens under high ambient temperature. Italian Journal of Animal Science. 2018;17(3):804-814.
- Jha R, Das R, Oak S, Mishra P. Probiotics (direct-fed microbials) in poultry nutrition and their effects on nutrient utilization, growth, laying performance and gut health. Animals. 2020;10(10):1863-1879.
- Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Frontiers in Immunology. 2014;5(4):461-476.
- Khalid A, Khalid F, Mahreen N, Hussain SM, Shahzad MM, Khan S, Wang Z. Effect of spore-forming probiotics on poultry production: a review. Food Science of Animal Resources. 2022;42(6):968-986.
- Mazanko MS, Gorlov IF, Prazdnova EV, Makarenko MS, Usatov AV, Bren AB, Chikindas ML. *Bacillus* probiotic supplementations improve laying performance, egg quality, hatching of laying hens, and sperm quality of roosters. Probiotics and Antimicrobial Proteins. 2018;10(5):367-373.
- Pan X, Cai Y, Kong L, Xiao C, Zhu Q, Song Z. Probiotic effects of *Bacillus licheniformis* DSM5749 on

- growth performance and intestinal microecological balance of laying hens. *Frontiers in Nutrition*. 2022;9(2):868-903.
7. Saksrithai K, King AJ. *Lactobacillus* and dietary sunflower meal supplementation in layer diets: effects on specific serum content and hydrogen sulphide concentration in layer manure. *Research in Veterinary Science*. 2019;122(4):64-71.
 8. Xiang Q, Wang C, Zhang H, Lai W, Wei H, Peng J. Effects of different probiotics on laying performance, egg quality, oxidative status, and gut health in laying hens. *Animals*. 2019;9(12):1110-1126.