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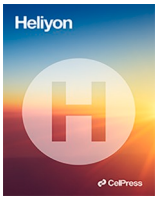

Research article

Dietary jack bean (*Canavalia ensiformis* L.) supplementation enhanced intestinal health by modulating intestinal integrity and immune responses of broiler chickens

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ABSTRACT

This study investigated the influence of supplementing with jack beans on jejunal morphology, cecal short-chain fatty acids production, gene expression both of pro- and anti-inflammatory cytokines and tight junctions. Four treatment groups including 288 Indian River chicks that were one day old were randomized at random. While the treatment groups received jack bean supplementation at levels of 5 %, 10 %, and 15 %, the control group (0 %) was given a basal diet. For 11–35 days, each treatment consisted of 8 pens with 9 birds each. Supplementing with jack beans significantly enhanced butyrate production ($P < 0.001$), while at 10 % supplementation did not differ from control. Villus height (VH) and the ratio (VH:CD) were significantly ($P < 0.001$) increased by dietary treatments, while villus width (VW) and crypt depth (CD) were significantly ($P < 0.05$) decreased. *TLR-3, TNF-a,* and *IL-6* were all significantly ($P < 0.001$) increased by dietary supplementation. However, at 15 %, *TLR-3* and *IL-6* were same with control. *IL-18* was significantly ($P < 0.05$) decreased at 15 %. *IL-10* decreased significantly ($P < 0.001$), but at 10 % same with control. At 5 and 10 %, *IL-13* increased significantly ($P < 0.001$), whereas dietary treatments decreased at 15 % compared to control. Although *ZO1* decreased significantly ($P < 0.001$) and *OLCN* increased significantly ($P < 0.001$), both *ZO1* and *OCLN* were not significantly different from the control at 15 %. Dietary treatments significantly ($P < 0.001$) increased *CLDN1* but did not differ from the control at 10 %. *JAM2* decreased significantly ($P < 0.001$) with dietary treatments. In conclusion, jack bean supplementation may increase broiler chicken performance and intestinal health due to butyrate production. It may affect intestinal morphology and integrity by upregulating a tight junction protein gene. Jack beans also impacted jejunum immune responses and inflammatory cytokine gene expression.

1. Introduction

Legumes intended for feeding have a comparatively high protein and carbohydrate content. The legumes typically have high levels of fat, fiber, minerals, vitamins, and crude oil. Furthermore, the presence of antinutrients limits the digestion of legume protein and starch [1], and all of these limitations restrict the use of legumes in animal nutrition. According to Nastiti [2], one indigenous bean that

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is known to have natural resistant starch (RS) is the jack bean. About 24.7–36.9 % of it is starch, of which 10.8 % is resistant starch and 26.1 % is digestible starch [3].

Resistant starch (RS) is the portion of starch that is not broken down by α-amylase in the small intestine but is still soluble due to its ability to be fermented by colon microflora, which produces short-chain fatty acids (SCFA) [4]. One kind of dietary fiber that resists digestion in the small intestine and makes it past the large intestine is resistant starch. Short-chain fatty acids including butyric acid, propionic acid, and acetic acid are produced in the large intestine by fermentation by the gut microbiota [5]. According to Mirzaei [4], this process influences immunological function [6] and has a prebiotic effect on intestinal health. It also improves the integrity of the intestinal epithelial barrier [7].

The first line of defense in the intestine is a healthy gut, which is maintained by the barrier function of epithelial cells [8]. To maintain gut integrity, the epithelial barrier keeps the host's gut luminal contents apart. It is commonly known that the most crucial elements for preserving the integrity of the intestinal barrier are SCFA, especially butyrate [9]. Furthermore, by improving tight junction protein and stimulating AMP-activated protein kinase (AMPK) in Caco-2 cell monolayers, SCFAs can control the inflammatory response and strengthen the integrity of the intestinal barrier [10,11]. By increasing the gene expression of the intestinal tight junction proteins *occludin*, *zonula occludens*, and *claudin-1*, SCFA can also preserve the integrity of the mechanical barrier [12].

Butyrate, a product of SCFA, has the ability to inhibit the synthesis of pro-inflammatory mediators such as *TNF-α*, *IL-6*, and *NO* [13]. Moreover, SCFAs could mitigate systemic inflammation by substantially lowering *TNF-α* and *IL-6* production via the downregulation of HDAC mRNAs [14]. By first eliminating pathogens through the secretion of inflammatory cytokines, the immune system defends the body against infections. On the other hand, diseases and systemic inflammation result from overproduction of cytokines [15]. By controlling the synthesis of cytokines and immune cell functions, SCFA have anti-inflammatory effects [16,17].

However, jack bean is a legume that contains numerous anti-nutrient factors, including enzyme inhibitors, phytic acid, flatulence factors, saponins, lectins, tannins, and glucosides [3]. Anti-nutrients may interfere with the absorption of nutrients, decrease digestibility, and even cause neurotoxic consequences when ingested in large amounts [18]. Thus, the objective of this study was to investigate the impact of different dietary doses of jack bean RS on intestinal morphology, cecal SCFA production, and gene expression both of pro- and anti-inflammatory cytokines and tight junction of broilers.

2. Materials and methods

All protocols relating to the care and use of live animals were reviewed and approved by Research Ethics Committee at the Faculty of Veterinary Medicine, Universitas Gadjah Mada, No. 036/EC-FKH Eks./2023.

Table 1
Chemical composition of jack bean seeds.

Chemical content	Results
Proximate analysis	
Dry matter, %	89.91
Crude ash, %	3.53
Crude protein, %	31.63
Crude fiber, %	9.13
Ether extract, %	3.01
Starch, %	28.45
Amino acid analysis	
Histidine, %	0.24
Serine, %	0.21
Arginine, %	0.13
Glycine, %	0.25
Aspartic acid, %	0.33
Glutamic acid, %	0.22
Threonine, %	0.11
Alanine, %	0.50
Proline, %	0.10
Lysine, %	0.24
Tyrosine, %	2.40
Valine, %	0.65
Isoleucine, %	0.14
Leucine, %	0.47
Phenylalanine, %	0.28
Cysteine, %	1.43
Methionine, %	0.26
Tryptophan, %	0.16
Anti-nutrient content	
Tannin, %	0.46
Phytic acid, g/100g	2.31
Hydrogen cyanide, mg/100g	6.24

2.1. Birds and housing

In this study, 300 Indian River strain broilers that had been vaccinated against Gumboro and Newcastle Disease (ND) in the hatchery were placed in brooding cages for ten days. On day 11 of the experiments, total 288 chickens were weighed and afterwards placed in colony cages. There were 32 cages in total inside the 1 × 1 m colony cages. Total 9 chickens, a 3:1 ratio of male to female, were housed in each cage with weighed 2430 ± 5 g/pen as initial body weight. Each cage is equipped with a feeder tray and automatic nipple drinker. The broiler management procedures followed the guidelines described in the Aviagen Indian Riven broiler management guidebook [19]. After being maintained at 30 °C for three days, the temperature was lowered by 2.5 °C each week until it reached 20 °C. For the first seven days, early-stage lighting patterns provide a lengthy day with 23 h of light and 1 h of darkness. It could be better to have four or 5 h of darkness after seven days.

Table 2
Compositions and nutrient content of experimental grower (11–21 d) and finisher (22–35 d) diets.

Feed ingredient	Percentage (%)							
	Grower (11–21 d)				Finisher (22–35 d)			
	0 %	5 %	10 %	15 %	0 %	5 %	10 %	15 %
Corn	59.51	57.81	55.64	53.90	59.86	58.35	57.00	55.60
Rice bran	7.15	7.38	8.03	8.34	9.69	9.70	10.05	10.42
Soybean meal	23.87	19.84	15.90	11.80	19.85	15.83	11.57	7.28
Meat bone meal	5.00	5.00	5.00	5.00	4.00	4.00	4.00	4.00
Jack bean	0.00	5.00	10.00	15.00	0.00	5.00	10.00	15.00
Crude palm oil	1.50	1.50	1.50	1.50	3.50	3.50	3.20	2.90
Limestone	0.46	0.48	0.49	0.51	0.58	0.58	0.58	0.59
Di-Calcium phosphate	0.55	0.55	0.54	0.54	0.55	0.55	0.55	0.56
NaCl	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Vitamin mix[a]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Mineral mix[b]	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Choline chloride	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.10
Toxin Binder[c]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
L-Lysine HCL	0.35	0.50	0.64	0.78	0.35	0.50	0.65	0.80
DL-Methionine	0.35	0.32	0.29	0.27	0.33	0.30	0.28	0.25
L-Threonine	0.14	0.23	0.32	0.41	0.11	0.20	0.30	0.40
L-Tryptophan	0.00	0.00	0.00	0.00	0.00	0.02	0.04	0.06
L-Arginine	0.00	0.15	0.28	0.45	0.04	0.19	0.34	0.50
L-Valine	0.05	0.11	0.18	0.25	0.05	0.11	0.18	0.25
L-Isoleucine	0.08	0.14	0.20	0.26	0.10	0.18	0.27	0.39
Calculated composition (%)								
Crude protein	21.95	21.95	21.96	21.97	19.77	19.79	19.77	19.77
ME (Kcal/kg)	3152	3164	3171	3184	3259	3274	3275	3276
Ca	0.87	0.87	0.87	0.87	0.81	0.80	0.80	0.79
Total P	0.74	0.73	0.72	0.71	0.71	0.69	0.68	0.67
Avail. P	0.47	0.46	0.45	0.44	0.42	0.41	0.40	0.40
Ca:P	1.88	1.91	1.93	1.97	1.93	1.96	1.98	2.00
Na	0.18	0.18	0.18	0.18	0.18	0.17	0.17	0.17
Cl	0.31	0.37	0.43	0.48	0.31	0.36	0.42	0.48
Choline	0.18	0.17	0.16	0.15	0.18	0.17	0.16	0.15
Lysine	1.29	1.29	1.29	1.29	1.16	1.16	1.16	1.16
Methionine	0.65	0.61	0.56	0.53	0.61	0.56	0.53	0.48
Methionine + Cysteine	0.99	0.98	0.98	0.98	0.91	0.91	0.91	0.91
Isoleucine	0.89	0.89	0.89	0.89	0.81	0.83	0.85	0.91
Threonine	0.88	0.88	0.88	0.88	0.78	0.78	0.78	0.78
Tryptophan	0.23	0.21	0.19	0.17	0.20	0.20	0.20	0.20
Arginine	1.35	1.34	1.33	1.34	1.23	1.23	1.22	1.22
Valine	1.00	1.00	1.00	1.00	0.91	0.90	0.90	0.90
Proximate analysis								
Dry matter	87.33	87.89	88.22	88.10	89.66	89.48	89.68	90.16
Crude protein	22.93	22.62	22.81	22.82	20.64	20.61	20.65	20.53
Crude fiber	3.48	4.85	4.54	4.98	4.95	5.06	5.57	5.70
Ether extract	4.74	4.88	4.88	4.71	6.77	7.41	7.95	7.56
Crude ash	7.28	7.45	7.14	8.06	9.32	8.31	7.06	6.47
Gross energy (Kcal/g)	3868	3907	3902	3880	4048	4035	4083	4069

[a] Supplied per kg of diet: vitamin A, 50,000,000 IU; vitamin D3, 10,000,000 IU; vitamin E, 80,000 mg; vitamin K3, 10,000 mg; vitamin B1, 10,000 mg; vitamin B2, 30,000 mg; vitamin B3, 225,000 mg; vitamin B5, 62,000 mg; vitamin B6, 10,000 mg; vitamin B9, 5000 mg; vitamin B12, 100 mg; vitamin H, 100 mg; vitamin C, 20,000 mg.

[b] Supplied per kg of diet: Mn, 40,000 mg; Fe, 32,000 mg; Cu, 6050 mg; Zn, 32,000 mg; I, 404 mg; Se, 100 mg.

[c] Supplied from Mycofix® Biomin.

2.2. Experimental design and diets

The current study used a 1-way pattern design, which is a completely randomized design. The treatment were divided into four groups, and each group's bird received a basal-based diet (0 %) supplemented with 5 %, 10 %, and 15 % jack Bean (*Canavalia ensiformis* L.). There were eight replications of each treatment, with three female and six male animals in each. Jack bean was obtained from local farmers at Pamekasan, East Java, Indonesia. The seeds were stored at cold chiller temperature after being ground to fit through a 0.5 mm sieve. The chemical composition of the seeds is presented in Table 1. Feeding treatments were given from the age of 11–35 days. The starting phase (1–10 days) was fed commercial feed, whereas the growth phase (11–21 days) and finisher phase (22–35 days) were fed according to Aviagen guidelines [20], which are presented in Table 2. Water and feed are freely available ad libitum.

2.3. Sample collections and preparations

At the end of the experiment (35 d), total 32 birds with 8 per treatment and 1 per replicate with BW closed to the median for each group, were chosen, weighed, and slaughtered by decapitating and severing the jugular vein using the halal methods. The middle part of the jejunum was cut about 2 cm and placed in the tube which contains 10 % buffered formalin solutions for jejunal morphology analysis. The jejunum sample from each bird was collected in a microtube. The microtubes immediately frozen in liquid nitrogen and stored at −80 °C until analyzed for gene expression of inflammation and tight junction. The contents of the cecum were collected in a microtube aseptically and stored −80 °C until analyzed for SCFA.

2.4. Cecal digesta SCFA profiles

Short-Chain Fatty Acids (SCFA) was determined as described by Liao [21], a 0.5 g sample of intestinal contents was suspended in 1.5 mL 2.5 % metaphosphoric acid solution. The suspensions were placed in ice water for 30 min immediately, homogenized with a vortex intermittently, and then centrifuged for 10 min at 14,000 g at 4 °C. The supernatant was used to determine the concentrations of SCFAs using gas chromatography (Ailgent 780A, Wilmington, NC).

2.5. Jejunal morphology

The jejunum samples were analylis according to Li [22]. Briefly, the tissue samples of jejunum and ileum fixed with 4 % para-formaldehyde were decalcified with decalcification solution, dehydrated with ethanol, transparent with xylene, and embedded in paraffin. Each tissue was cut into 3 sections with well-oriented parts using a Leica RM 2235 microtome, then dewaxed with xylene and stained with H&E. Microscope images were taken at 40X (Nikon, Tokyo, Japan). The villus height (VH), villus width (VW), and crypt depth (CD) of jejunum and ileum were measured and recorded by Image-Pro Plus 6.0 software, and the VH/CD was calculated.

Table 3
Primer pairs for analysis of inflammation and tight junction gene expression.

Gene	GeneBank accession No.	Primer sequence (5'→3')	Orientation	Product size (bp)
β-actin		GTGTGATGGTTGGTATGGGC	Forward	225
		CTCTGTTGGCTTTGGGGTTC	Reverse	
TLR-3	NM_001011691.3	GATTGCACCTGTGAAAGCATTG	Forward	67
		CGGGTATATATGCTTGAGTGTCGTT	Reverse	
TLR-4	NM_001030693.1	TCCTCCAGGCAGCTATCAAGAT	Forward	74
		GACAACCACAGAGCTCATGCA	Reverse	
TNF-α	NM_204267	CGTTTGGGAGTGGGCTTTAA	Forward	61
		GCTGATGGCAGAGGCAGAA	Reverse	
IL-18	GU119895	TGCAGCTCCAAGGCTTTTAAG	Forward	63
		CTCAAAGGCCAAGAACATTCCT	Reverse	
IL-6	NM_204628.1	GCTTCGACGAGGAGAAATGC	Forward	63
		GGTAGGTCTGAAAGGCGAACAG	Reverse	
IL-10	AJ621614	CATGCTGCTGGGCCTGAA	Forward	63
		CGTCTCCTTGATCTGCTTGATG	Reverse	
IL-13	AJ621735	CCAGGGCATCCAGAAGC	Forward	256
		CAGTGCCGGCAAGAAGTT	Reverse	
ZO1	XM_015278975	GCCAACTGATGCTGAACCAA	Forward	141
		GGGAGAGACAGGACAGGACT	Reverse	
CLDN1	NM_001013611	GGTGAAGAAGATGCGGATGG	Forward	137
		ATCGCCCTGTCCGTCATC	Reverse	
JAM2	XM_015299112	CTGCTCCTCGGGTACTTGG	Forward	135
		CCCTTTTGAAAATTTGTGCTTGC	Reverse	
OCLN	NM_205128	GATGGACAGCATCAACGACC	Forward	142
		CTTGCTTTGGTAGTCTGGGC	Reverse	

2.6. Gene expression by RT-qPCR assay

Jejunal samples were obtained in a microtube from six bird in each treatment. Microtubes were instantly frozen in liquid nitrogen and kept at −80 °C until analyzed. The gene expression analysis begins with RNA extraction from a 20 mg jejunum sample using a Quick-RNA minirep kit (R1054 model) Zymo Research (Orange, California) according to instructions. The Nanodrop Spectrophotometer (Maestrogen Inc, Hsinchu City, 30091, Taiwan) was used to determine the purity and amount of RNA. Using ReverTrace qPCR RT Master Mix (Toyobo), whole RNA was employed as a template for cDNA synthesis using reverse transcriptase enzyme. According to the protocol, relative gene expressions were measured using a QuantStudio 3 Real-Time PCR machine (Thermo Fisher Scientific) using Thunderbird SYBR qPCR Mix (Toyobo). In a 20 μL reaction volume containing nuclease-free water, 2 μL diluted cDNA, 6 pmol forward primer, 6 pmol reverse primer, 0,04 μL ROX reference dye, and 10 μL qPCR Mix were poured to the tube.

Table 3 contains all primer pairs used for Toll-Like Receptor-3 (*TLR-3*), and Toll-Like Receptor-4 (*TLR-4*), Interleukin-6 (*IL-6*), Interleukin-10 (*IL-10*), Interleukin-13 (*IL-13*), Interleukin-18 (*IL-18*), Tumor Necrosis Factor-alpha (*TNF-α),* Zonula Occludens-1 (*ZO1*), Claudin-1 (*CLDN1*), Junctional Adhesion Molecule-2 (*JAM2*), and Occludin (*OCLN*). The following amplification schedule was used: a hold stage at 95 °C for 2 min, followed by a PCR stage at 95 °C for 1 s and 60 °C for 30 s. The melt curve was examined to identify product amplification at the end of the run. Each group received eight samples, with each sample being conducted in duplicate. The $2^{-\Delta\Delta CT}$ technique was used to normalize the mRNA levels as a ratio to β-actin in random units, and the data were reported as relative values to the control group [23].

2.7. Statistical analyses

All experimental data were analyzed statistically using IBM SPSS statistic version 26.0. the data subjected to one-way ANOVA among 4 treatments. A Duncan test was used to determine significant differences among all treatments. The statistical significance of all analyses was set at $P < 0.05$ for probability values.

3. Results

3.1. SCFA production

Dietary supplementation of jack bean on SCFA production were shown in Table 4. Dietary supplementation of jack bean increased butyrate production significantly ($P < 0.001$), but at 10 % level of supplementation didn't different compared to the control. The jack bean supplementation on broiler feed did not influence significantly on acetic acid, propionic acid, isobutyric acid, isovaleric acid, and total SCFA production.

3.2. Jejunal morphology

The effects of dietary treatments on jejunal morphology were presented in Table 5. Supplementing the diet with jack bean leads to a significant increase ($P < 0.001$) in both villus height (VH) and the VH:CD ratio. The addition of jack bean to the broiler diet resulted in a significant decrease ($P < 0.05$) in both villus width (VW) and crypt depth (CD).

3.3. Expression of tight junction and inflammatory cytokines genes

The dietary jack bean supplementation on expression influence of tight junction (Fig. 1), toll-like receptor (Fig. 2), pro-inflammatory cytokines (Fig. 3) and anti-inflammatory cytokines (Fig. 4). Tight junction gene expression of *ZO1* and *JAM2* decrease significantly ($P < 0.001$) by the dietary jack bean treatment, but *ZO1* at 15 % level didn't differ compared to the control. *CLDN1* and *OCLN* increased significantly ($P < 0.001$), but both *CLDN1* (at 10 %) and *OCLN* (at 15 %) were didn't different compared to control For *TLR* gene expression, *TLR-3* increased significantly ($P < 0.001$), but at 15 % level of supplementation did not different compared with control. Pro-inflammatory gene expression in comparison with the control, the broiler jejunal mRNA expression of the *TNF-α* and *IL-6* were increased significantly ($P < 0.001$) by dietary jack bean treatment, but *IL-6* at 15 % was same with control.

Table 4
Effect dietary jack bean supplementation on SCFA production ($n = 8$ per treatment).

Parameters	Treatments				SEM	*P*-value
	0 %	5 %	10 %	15 %		
Acetic acid, mg/L	2057.48	2415.88	2202.59	2132.13	65.40	0.250
Propionic acid, mg/L	341.28	384.25	387.62	432.47	19.21	0.444
Butyric acid, mg/L	313.83[b]	529.13[a]	356.16[b]	579.67[a]	30.67	<0.001
Isobutyric acid, mg/L	53.61	69.35	69.66	66.55	3.28	0.262
Isovaleric acid, mg/L	34.07	45.34	48.13	38.20	3.44	0.475
Total SCFA, mg/L	2749.67	3274.00	3027.97	3150.37	106.03	0.357

[a,b] Means within a column with different superscripts are different ($P < 0.05$). Abbreviations: SEM, Standard error of the mean.

Table 5
Effect dietary jack bean supplementation on jejunal morphology ($n = 6$ per treatment).

Parameters	Treatments				SEM	*P*-value
	0 %	5 %	10 %	15 %		
Villus height, μm	897.85[b]	1477.63[a]	1412.70[a]	1433.23[a]	52.47	<0.001
Villus width, μm	331.08[a]	185.16[b]	164.67[b]	231.52[b]	18.44	0.002
Crypt depth, μm	199.85[a]	159.00[b]	157.35[b]	154.30[b]	6.91	0.050
Villus wide area, μm^2	255798.59[a,b]	215974.85[b]	230403.28[b]	309756.62[a]	11540.49	0.012
Ratio VH:CD	4.84[b]	9.31[a]	8.97[a]	9.36[a]	0.44	<0.001

[a,b] Means within a column with different superscripts are different ($P < 0.05$). Abbreviations: SEM, Standard error of the mean.

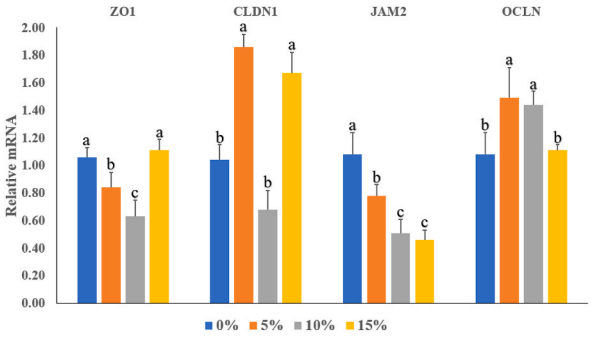

Fig. 1. Comparison between gene expression tight junction zonula occludens-1 (*ZO1*), claudin-1 (*CLDN1*), junctional adhesion molecule 2 (*JAM2*), and occludin (*OCLN*) of broiler chicken by dietary jack bean supplementation on different level (n = 6 per treatment).

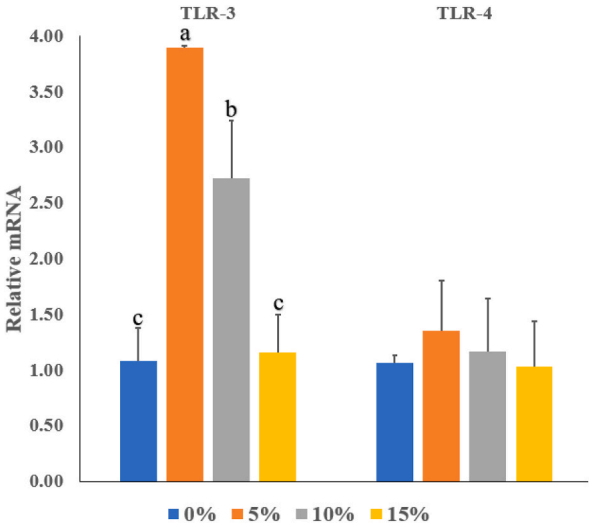

Fig. 2. Comparison between gene expression toll-like receptor-3 (*TLR3*) and toll-like receptor-4 (*TLR4*) of broiler chicken by dietary jack bean supplementation on different level (n = 6 per treatment).

Meanwhile, *IL-18* was decrease significantly ($P < 0.05$) at 15 % level of supplementation. Anti-inflammatory, *IL-10* was decreased significantly ($P < 0.001$), but at 10 % same with control. On the other hand, dietary jack bean supplementation increased significantly ($P < 0.001$) on *IL-13*, but 15 % was lower compared to other treatments.

4. Discussion

4.1. Cecal digesta SCFA profiles

This study shows that the addition of jack bean to the diet leads to an increase in the generation of butyrate. The Jack bean contains both starch and resistant starch (RS) [3]. Microorganisms ferment resistant starch in the large intestine [24]. Resistant starch takes on

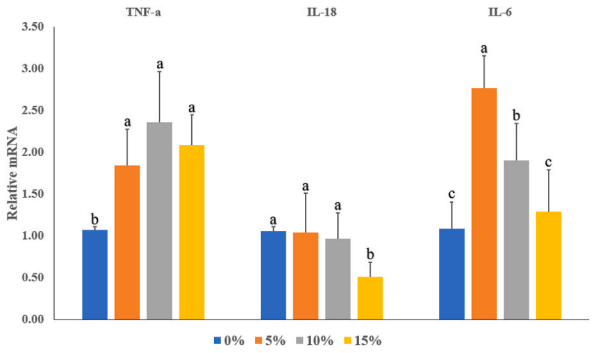

Fig. 3. Comparison between gene expression pro-inflammatory cytokines tumor necrosis factor-α (*TNF-α),* interleukin-6 (*IL-6*), and interleukin-18 (*IL-18*) of broiler chicken by dietary jack bean supplementation on different level (n = 6 per treatment).

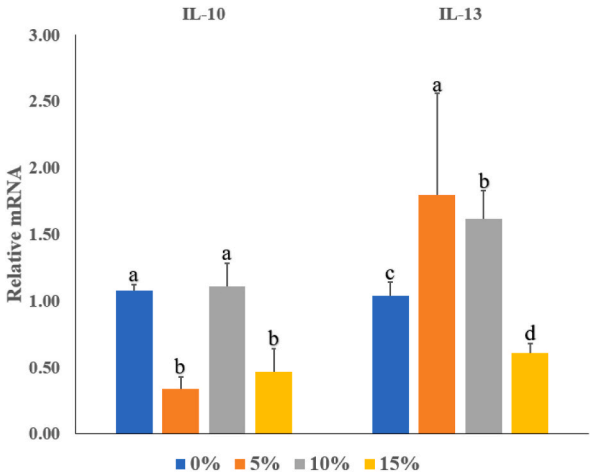

Fig. 4. Comparison between gene expression anti-inflammatory cytokines interleukin-10 (*IL-10*), and interleukin-13 (*IL-13*) of broiler chicken by dietary jack bean supplementation on different level (n = 6 per treatment).

fermentation in the cecum and colon, resulting in the production of SCFA [25,26]. Several research studies have documented that resistant starch yields the highest levels of butyrate in comparison to other types of dietary fibers [27,28]. Butyrate, a type of SCFA, is added to chicken diets as a feed supplement to enhance intestinal health, performance, and immunity [29]. Short-chain fatty acids can serve as both an energy and carbon source for poultry [30,31]. Butyrate promotes rapidly changing to butyric acid in the poultry digestive system, leading to enhanced intestinal health through many mechanisms [32,33]. Butyrate in poultry serves several functions, including reducing the pH of the intestine to promote the growth of beneficial bacteria like *Lactobacilli* and *Bifidobacteria* spp. It also enhances the development of gut wall tissues, regulates the growth of symbiotic intestinal microflora, boosts immunity in broilers, and enhances the integrity of the epithelial cell layer, which is crucial for normal intestinal function [34–36].

In a recent study conducted by Qin [37], it was found that the inclusion of resistant starch (RS) in the diet of ducks resulted in a significantly increase in butyrate from the cecal digesta. The increased levels of SCFA, particularly butyrate, detected in the large intestine are in line with findings in pigs [38] and rats [39]. Hedemann and Bach Knudsen [40] found that including resistant starch (RS) in the diet significantly increased the production of butyrate. Previous studies have consistently shown that RS cannot be broken down by enzymatic digestion in the small intestine. However, it undergoes extensive fermentation by the microbiota in the colon, leading to the production of SCFA. This finding has been supported by various studies [40–44]. In this finding, the dietary supplementation of jack on broiler feed at 10 % did not difference butyrate production. It might be due to the contain of several anti-nutrients in jack bean. On the other hand, *claudin-1* (Fig. 1) showed the statistically significant effect at 10 % same as butyrate production, as indicated the relate situation. These results will provide new information on the use of jack bean in broiler diets.

4.2. Jejunal morphology

The present study found that adding jack bean to the diet resulted in an increase in villous height (VH) and the VH:CD ratio. Resistant starch found in jack bean is not broken down in the small intestine but is instead metabolized by bacteria in the hindgut. These microbes ferment the starch, producing SCFA such as acetic acid, propionic acid, and butyric acid [45]. Out of the SCFA,

butyrate has been widely studied. According to Roediger [46], butyrate is the primary energy source for the cells in the colon known as colonocytes. Villus height and crypt depth in the small intestine were utilized as measures of intestinal health, as they are both markers of efficient nutrition absorption [47]. Several previous studies have demonstrated that butyric acid promotes the formation of intestinal villi [48–52]. The potential mechanism by which sodium butyrates improves intestinal health is through the stimulation of intestinal blood flow and production of gastrointestinal hormones by butyrate [53].

According to recent research by Qin [37], jack bean contains resistant starch, which has been demonstrated to have a positive impact on the intestinal structure of ducks when compared to purified raw potato starch. The results were contrary to the findings of Zhang [54], who discovered a substantial loss in jejunal morphology in all treatments due to resistant starch. The primary factor may be the contrasting types of root systems found in jack bean and corn. Jack bean starch exhibits distinct characteristics compared to maize starch, including enhanced stability and increased resistance to α-amylase bacterial activity [55]. Furthermore, a decrease in the height of the villus would result in a reduction in the surface area available for nutritional absorption. Research has demonstrated that butyrate has the ability to enhance the ratio of villus height to crypt depth (VH:CD) in broilers. This ratio serves as a significant indicator of intestinal health and function [56–58]. The VH:CD ratio, which stands for the ratio of villi height to crypt depth, has a crucial role in both nutrient absorption and gut health [58].

The inclusion of jack bean in the diet had an impact on the dimensions of the villi in broiler chickens, specifically their length and width. The dietary treatment resulted in a decrease in villus width (VW) and crypt depth (CD) in this study. This discovery aligns with the findings of Qin [37], who observed a substantial decrease in CD in ducks that were fed with RS. The existence of anti-nutritional elements like lectins and protein amylase inhibitors have a significant impact on the digestion of jack bean starch [59]. According to these scientists, Con A might hinder the function of pancreatic amylase by directly engaging with the enzyme structure or the starch molecule. This connection leads to the formation of a protein crust that impacts the interaction between carbohydrates and enzymes, ultimately slowing the hydrolysis process.

4.3. Expression of tight junction and inflammatory cytokines genes

Butyrate is acknowledged as the primary force behind maintaining the integrity of the intestinal barrier. It regulates the growth, specialization, and programmed cell death of colon cells. The maintenance of the gut barrier is influenced by several mechanisms, as stated by Knudsen [60]. In the beginning, butyrate serves as the main source of nourishment for colon cells, which rely on the intestinal tract rather than the bloodstream for their sustenance [61,62]. Insufficient butyrate can lead to the formation of holes and leaks between epithelial cells. Furthermore, butyrate functions as an inhibitor of histone deacetylase (HDAC) and as an activator of G protein-coupled receptor (GPCR), among other roles, in regulating gene expression [63,64]. These pathways manage a multitude of genes that affect inflammation, immunity, hunger, and energy balance. It is widely considered that these pathways are responsible for the anti-carcinogenic, anti-inflammatory, and neuroprotective advantages of butyrate.

In this present study, the dietary jack bean treatments resulted in an increase in *TLR-3* expression. However, at a level of 15 %, there was no significant difference compared to the control group. Butyrate has the ability to modify *TLR* responses and possesses immunomodulatory properties that can affect the immune response in the gastrointestinal tract [65]. *TLRs* are not inherently pro-inflammatory molecules, but they do have a significant role in triggering and regulating inflammatory reactions. *TLRs* are a class of receptors found on immune cells that have the ability to identify chemicals originating from several types of pathogens, including bacteria and viruses. *TLRs* initiate signaling pathways within cells upon recognition and binding to bacterial or viral molecules, resulting in the subsequent generation and release of several pro-inflammatory cytokines. Subsequently, these cytokines will activate the inflammatory process as a component of the body's immune reaction to infection [66]. *TLRs* have crucial functions in innate immune responses since they detect different components produced from pathogens. When these receptors are activated, they trigger the production of inflammatory cytokines [67].

T cells are the primary source of cytokines, which might serve as an indicator of the body's immune response capacity [68]. A correlation exists between *TLRs* and T-helper (Th) cells. *TLRs* and Th cells play crucial roles in the detection and reaction to infections, and they have the ability to mutually influence immune responses [69]. Furthermore, Th cells have the ability to generate cytokines that control the expression and operation of *TLRs* [70]. Both of *TLR-3* and *TLR-4* are crucial in both humoral and cellular immunity, as well as in cytokine synthesis during immunological responses. They play a role in identifying harmful microorganisms and triggering the response of the body's natural defense cells, resulting in the release of cytokines and the formation of adaptive immunological reactions [71,72]. In this investigation, the dietary jack bean treatment resulted in an increase in *TNF-α* and *IL-6* levels. Nevertheless, there was no significant difference in *IL-6* levels at 15 % in comparison to the control group. In contrast, the dietary treatment resulted in a decrease in *IL-18* levels and the groups that were given 5 % and 15 % jack bean supplementation showed a decrease in the levels of mRNA expression for the anti-inflammatory cytokine *IL-10*, as compared to the control group.

The level of *IL-13* was reduced by 15 %, which was lower than both the control group and all other therapies. The results showed that the immune response, both at the cellular and humoral level, was increased in broilers that were fed a diet containing jack bean with resistant starch and underwent butyrate conversion during fermentation in the cecum. The presence of anti-nutritional elements, such as lectins and protein amylase inhibitors [59], significantly impact the digestion of jack bean starch.

The current study examined the effects of various dietary treatments of jack bean, which contains resistant starch, on the mRNA expression of *CLDN1* and *OCLN*. The results showed that varied levels of jack bean in the diet led to an increase in the mRNA expression of *CLDN1* and *OCLN*. Activation of TLRs can induce the synthesis of cytokines that control the expression of *CLDN1* and *OCLN* [73]. These proteins play a crucial role in regulating the permeability of the gut barrier [74]. In a study conducted by Qin [37], it was discovered that the inclusion of resistant potato starch in the diet of meat ducks enhanced the integrity of their intestinal barrier.

Specifically, they demonstrated that butyrate increased the expression of genes associated with the intestinal barrier, such as *CLDN-1*, *ZO-1*, *mucin-2*, and *proglucagon* in the cecum. These genes play a role in regulating the permeability of the intestines. In addition, tight junctions play a key role in determining the permeability of the intestines [75]. In addition to promoting the proliferation of intestinal epithelial cells, butyrate has also been shown to enhance the function of the gut barrier [11]. In this current investigation, the dietary jack bean treatment resulted in a decrease in the expression of *ZO1* and *JAM2*. The reduced digestibility of jack bean starch may be attributed to the presence of complex anti-nutrients [3], as well as lectins and protein amylase inhibitors [59]. It suggested the presence of a highly intricate mechanism that governs the connection between the jack bean, which contains resistant starch, and the operation of the intestinal barrier in broiler chickens. Possible factors could include either the particular nature of the digestive tract in poultry or the variation in susceptibility of ducks to resistant starch. Therefore, additional research is required to confirm this potential.

Overall, incorporating jack bean into the diet of broiler chickens has the capacity to enhance their performance and intestinal health by promoting the formation of butyrate. It has the potential to affect the structure and integrity of the intestines by increasing the expression of genes associated to tight junction proteins. Furthermore, the consumption of jack bean in the diet had an impact on the immune responses in the jejunum and the expression of genes related to inflammatory cytokines. Additionally, we recommend conducting additional processing of jack bean prior to including it into broiler feed in order to decrease the presence of anti-nutrients.

Ethical statement

This study was reviewed and approved by the Research Ethics Committee at the Faculty of Veterinary Medicine, Universitas Gadjah Mada, No. 036/EC-FKH Eks./2023.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Abd Majid Ahmad Madani: Writing – original draft, Project administration, Methodology, Investigation, Formal analysis. **Muhlisin Muhlisin:** Writing – original draft, Methodology, Data curation, Conceptualization. **Asih Kurniawati:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Aji Praba Baskara:** Methodology, Data curation, Conceptualization. **Muhsin Al Anas:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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.

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