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Research Article

Effects of Different Culture Media of Lactic Acid Bacteria on Performance, Carcass Yield, Blood Parameters, and Natural Antibodies in Broiler Chickens

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Abstract

An experiment was conducted to evaluate the effect of various culture media used to provide nutrients for lactic acid bacteria (LAB) on performance, carcass yield, blood parameters, natural antibody titers, and lipid and protein metabolism namely plasma triglycerides, total protein, albumin, and urea in broiler chickens. A total of 400 one-day-old Cobb broiler chicks was allocated to four treatments**:** a control feed (no probiotics), feed supplemented with 2% probiotic powder, which had been cultured in one of the following media: (1) 100% cow milk (CM), (2) a mixture of 50% cow milk and 50% soybean milk (SM), or (3) a combination of 50% cow milk, 25% soybean milk, and 25% mung bean milk (MM), reared for 28 days of experimental period. The results showed that, birds fed SM exhibited higher body weight, body weight gain, feed intake, and better feed conversion ratio than other groups, as well as higher carcass percentages, lower abdominal fat, and lower plasma and meat cholesterol. They also showed significantly lower plasma triglyceride, total protein, and albumin levels ($P<0.01$) and reduced IgY and IgM antibody titers binding keyhole limpet hemocyanin (KLH). Nonetheless, all treatments demonstrated an increasing immune response over time. These data indicate that a 50:50 cow milk and soybean milk probiotic culture could enhance broiler performance, carcass yield, plasma lipid metabolism, blood metabolite profiles (such as protein, albumin, and urea) and immune status.

Keywords: Antibody titers, Broiler, Carcass yield, Immunoglobulin Y, Lipid metabolism, Probiotic

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Introduction

It is known that using antibiotics in poultry production, both as immunomodulators and as growth promoter leads to bacterial resistance against antibiotics, which can also be transferred to humans [1,2]. Therefore, identifying effective alternatives to antibiotics is of growing importance in veterinary research and poultry production systems. One of the common alternatives is the use of probiotics; The growing global interest in probiotics is not only reflected in the expanding market size, but also in the sharp rise in academic publications over the past decade [3].

Probiotics are live microorganisms which when supplemented in adequate amounts confer a beneficial health effect on the host [1,4]. Most microorganisms that can perform such activities are lactic acid bacteria (LAB), such as *Bifidobacteria* and *Bacillus* strains [1]. Originally, probiotics were used for human and animal microbiota composition and environment modification, which can improve their health [5]. Currently, probiotics aim to result in multiple benefits, from nutrient absorption enhancement [6] to immunomodulatory effects [7,8]. Probiotics have been widely recognized for their positive impact on poultry immune status, health, and performance [9]. While other alternatives such as organic acids, enzymes, and phytogenics have also been explored for similar benefits [10] their practical application in commercial settings can be limited by factors such as cost, formulation complexity, or regulatory hurdles.

In the context of probiotic cultivation, ensuring the viability and metabolic activity of LAB is of paramount importance [11]. A conducive culture medium is integral to this, playing a pivotal role in imparting essential nutrients that foster the growth and metabolic vitality of the LAB [11,12]. Carbohydrate-rich solutions traditionally act as the milieu in which LAB exhibit optimal growth [12]. Despite its ubiquity, cow's milk as a LAB culture medium presents challenges, primarily owing to its limited proteolytic activity which often inhibits maximal LAB proliferation, thus prompting the exploration of alternative culture media [13]. Recent scientific explorations have opened avenues beyond traditional dairy media. A novel approach involves employing functional foods for direct LAB inoculation, positing a significant departure from conventional methodologies [14].

Extending this line of inquiry, our current research evaluates the potential of leguminous substrates, specifically soybean *(Glycine max)* and mung bean *(Vigna radiata),* as culture media of LAB. These legumes were selected due to their rich content of oligosaccharides, dietary fiber, and bioactive compounds and isoflavones and phytosterols and α-linolenic acid, which have been associated with hypocholesterolemic, anti-inflammatory, and immunomodulatory effects [15-17]. Soybean, in particular, has been shown to support microbial fermentation by enhancing the growth and metabolism of probiotic strains such as *Bifidobacterium animalis*, through the activity of its digested peptides and their regulatory effects on metabolic pathways like glycolysis and pyruvate metabolism [18]. Likewise, mung bean milk (MBM) has demonstrated strong potential as a LAB fermentation medium. Wu et al.[19] optimized fermentation conditions for *Lactobacillus plantarum* B1-6 in mung bean meal, achieving a high LAB count of 8.96 log CFU/mL, and noted significant protein hydrolysis (up to 64%) and ACE-inhibitory activity (67.5%), indicating health-promoting properties relevant to gut and metabolic health. Further studies have shown that *Lactococcus lactis* fermentation of mung bean milk improves antioxidant capacity, protein solubility, and sensory properties, while reducing anti-nutritional factors [20].

Altogether, these attributes make soybean and mung bean ideal candidates for probiotic media development, providing a sustainable, plant-based alternative to dairy substrates for LAB cultivation with potential added benefits for poultry health and performance. The academic discourse around plant-based yogurt alternatives, especially those derived from pulses, has witnessed a burgeoning interest. Despite being relatively nascent entrants in the commercial market, their physicochemical propensity to undergo gelation, mirroring the acid-induced gelation characteristic of dairy yogurts, is of particular interest to microbiologists [21-23]. The synergistic dynamics between specific thermal treatments and LAB strains, particularly those proficient in Exopolysaccharide (EPS) synthesis, offer intriguing prospects to enhance fermentation efficiency and optimize the textural attributes of the resultant product [24-26].

Prior to translating these microbiological findings to practical applications, it is imperative to quantitatively assess the viability of LAB cultivated in these alternative media using standard plate count methods (CFU/mL) and evaluating their acidification activity and survival rates over time under simulated gastrointestinal and storage conditions. Accurate enumeration of these viable microorganisms provides a critical metric for subsequent studies. In the current study, this foundational research was extended by administering probiotics to broiler chickens and evaluating not only growth performance but also health indicators, including blood profiles, lipid metabolism, and immunological responses, specifically, the natural antibody titer against the foreign chicken antigen keyhole limpet hemocyanin (KLH) [26].

Material and Methods

Ethical Statement

All procedures involving animals were conducted in accordance with ethical standards for the care and use of laboratory animals and were approved by the Ethical Committee of the Universitas Padjadjaran, Indonesia (approval number: 0718070998). The study adhered to national animal welfare regulations and followed the principles of the 3Rs (Replacement, Reduction, and Refinement) to minimize animal suffering. Birds were monitored daily for health and well-being, and all handling was performed by trained personnel to ensure humane treatment.

Probiotics Preparation

The preparation of the probiotics was based on the procedure described by Kumalasari et al.[27] and Nabila et al.[28]. The pasteurized cow milk used in this study was procured from a dairy farm cooperation (altitude: 1200 masl) in Bandung (KPSBU Lembang, Bandung, Indonesia), while the soybean and mung bean were obtained from the local commercial market (Bandung, Indonesia). The probiotics utilized came from yoghurt with a consortium of microbiota, which included 5% (v/v) of *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Bifidobacterium* sp. [27,29]. This blend was introduced to the de Man, Rogosa and Sharpe (MRS) medium (Merck, Darmstadt, Germany) and left to incubate at 45°C for 14 h. Post incubation, the probiotics were mixed into different culture media: 100% cow milk (CM), a 50% cow milk and 50% soybean

milk mixture (SM), or a combination of 50% cow milk, 25% soybean milk, and 25% mung bean milk (MM), followed by thorough homogenization. The blend was then fermented at room temperature for 14 h. To create an encapsulation, the fermented blend was combined with food grade DE 10-12 maltodextrin 5%, skimmed milk, and pure distilled water at a 1:2 ratio to the overall solution volume. Maltodextrin acted as a protective layer against potential damage from external conditions like intense temperature fluctuations found in processes like spray drying. The probiotics were then converted into powder form through a spray drying method at 160°C input temperature and 65-70°C output temperature. Probiotic powders were freshly prepared weekly and added to the basal feed at a concentration of 2% (w/w). Feed analyses were conducted on final pooled samples representative of each treatment formulation. After this, a viability assessment was conducted, resulting in the microbiota's viability in the probiotic powder aligning with the standard lactic acid bacteria count for yogurt, 1.6×10^7 CFU/g, as mentioned in the Indonesian Standardization Body for yoghurt production [30].

Animals, Experimental Design, Housing and Rations

In a completely randomized design, 400 1-day-old Cobb broiler chicks used in this experiment were obtained from a commercial hatchery (initial weight = 43±2 g), vaccinated at day 1 for Newcastle, Marek's, and Infectious Bursal Disease, and randomly allocated to 4 experimental dietary treatments with 4 replicates. The experiment was conducted over a 28-day period, beginning on day 1 (the day of arrival and initial vaccination) and continuing until day 28. This duration was chosen based on the standard commercial grow-out period for Cobb broilers, which typically ranges between 28 to 35 days. The 4-week duration was deemed sufficient to evaluate early growth performance, blood parameters, and immune responses relevant to the probiotic supplementation effects. Each treatment consisted of 100 broilers. Each replicate was allocated into 5 floor pens so that there were 20 broilers per pen. The birds were raised on wood shavings litter (approximately 5-7 cm thickness) with maintained humidity (max. 60%). The heat was provided by an automatic temperature controller (Temptron 616, AgroLogic, Israel). Ambient temperature was maintained at 29±1°C at the starter phase and 25±1°C for the rest of the experiment. A 23L:1D lighting program was applied during the whole experiment. The basal diets were formulated according to Cobb-Vantress nutritional guidelines [29], and then supplemented with probiotic powders in accordance with the respective dietary treatment groups (see Probiotics Preparation section for details). During the whole study period, the chicks had ad libitum access to water and feed. The experimental diets were without any commercial growth promoter additives. The chemical composition of basal diet is shown in *(Table 1)* and the chemical composition of each treatment diet is shown in *(Table 2)*. The probiotics added to the basal feed were:

Table 1. *Composition of the basal diet used in this study*

Ingredients	Content (%)
Corn	57.30
Wheat bran	4.50
Soybean meal	27.00
Fish meal	7.60
Coconut oil	2.00
Meat bone meal	1.50
Methionine	0.10
Total	100

Table 2. *Chemical analysis of the treatment diet during the trial*

Item	CT	CM	SM	MM
Metabolize energy (ccal/kg)	3071.6	3129.47	3103.46	3106.10
Dry matter (%)	91.49	93.27	93.23	93.65
Crude protein	21.40	21.75	21.64	21.68
Crude fiber	4.62	4.73	4.75	4.80
Ether extract	8.21	8.42	8.32	8.32
Lysine	1.29	0.65	0.65	0.65
Methionine	0.51	0.98	0.98	0.98
Methionine + Cysteine	0.65	0.65	0.65	0.65
Calcium	0.98	0.98	0.98	0.98
Phosphorus	0.66	0.65	0.65	0.65

CT: *control (no probiotics supplementation);* ***CM:*** *basal ration + 2% of probiotics cultured in cow milk (100%) medium;* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean emulsion (50%) media;* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean emulsion (25%) media*

CT: control basal feed (no probiotics supplementation)

CM: basal feed + 2% (w/w) of probiotic powder ($4.51x10^3$ CFU/g)/kg of feed cultured in 100% cow milk

SM: basal feed + 2% (w/w) of probiotic powder ($3.85x10^3$ CFU/g)/kg of feed cultured in 50% cow milk and 50% soybean milk

MM: basal feed + 2% (w/w) of probiotic powder ($7.09x10^2$ CFU/g)/kg of feed cultured in 50% cow, 25% soybean, and 25% mung bean milk.

Performance parameters such as body weight gain, feed intake, feed conversion ratio, and mortality) were recorded each week from day 1 to day 28. Blood samples were collected on randomly selected birds within each treatment group at day 1, day 14, and day 28 (i.e., once

every two weeks). These samples were used to determine plasma metabolites and natural antibody titers, in accordance with the bi-weekly sampling schedule. Weight of bursa fabricius, abdominal fat, carcass percentages and meat samples were recorded and collected at the end of the treatment.

Blood and Meat Collection

Blood samples were collected from the pectoral vein at days 0, 14, and 28 during the early morning, with feed and water available ad libitum. Blood was collected in evacuated tubes (BD Vacutainer, Plymouth, UK) containing K2 EDTA (to obtain plasma) for plasma metabolites and natural antibody levels. Blood was collected in evacuated tubes containing K2 EDTA to obtain plasma, which was used for the analysis of plasma metabolites and natural antibody titers. EDTA plasma was selected due to its compatibility with the commercial assay kits and ELISA procedures used in this study. After collection, blood samples were kept on ice (maximum 2 h) to minimize cellular metabolism and preserve plasma integrity, until it centrifugated at 3000 x g (approximately 5000 rpm) for 15 min at room temperature. Plasma was decanted, aliquoted, and frozen at -20°C until analysis.

Meat collection has been described in our earlier study [27]. In brief, meat samples were taken at the end of the treatment period. A small portion of the chicken from the breast ± 5 g as a diced sample. Samples were kept frozen at -20°C until analysis.

Determinations of Blood Parameters

Determination of plasma triglycerides, total protein, cholesterol, urea, and albumin level was performed at the laboratory of Animal Physiology and Biochemistry, Universitas Padjadjaran (Bandung, West Java, Indonesia), using spectrophotometric analysis with commercial kits (Biolabo, Maizy, France) with the catalogue number of 80019, 80016, 80106, 80221, and 80002, respectively.

Determinations of Natural Antibodies (NAbs) Titers Against Keyhole Limpet Hemocyanin (KLH)

Determination of natural antibodies (Nabs) levels binding KLH were performed in the laboratory of Pharmacology and Biochemistry, Faculty of Pharmacy, Universitas Padjadjaran using an indirect enzyme-linked immunosorbent assay (ELISA) as described by Mayasari et al.[31]. Levels of NAb titers were determined by ELISA against KLH, a foreign antigen originating from *Megathura crenulata*, a keyhole limpet that lives off the coast of California, USA. This antigen was intended to reduce false positives in detecting NAb levels because the keyhole limpet is a gastropod, phylogenetically distant from avian proteins. Briefly, plates were coated with 4 µg/mL of KLH (Cat No. H8283, Sigma-Aldrich Merck, Darmstadt, Germany) in 100 µL/well. Natural antibodies of the IgY isotype were detected using a 1:20,000 dilution of Rabbit Anti-chicken IgY-heavy and light chain antibody conjugated to horse radish peroxidase (HRP) (Cat. No. A30-107A, Bethyl Laboratories, Montgomery, TX, USA), while levels of the IgM isotype were detected using a 1:20,000 dilution of Rabbit Anti-chicken IgM Antibody conjugated to HRP (Cat. No. A30-102P, Bethyl Laboratories, Montgomery, TX, USA). After washing the plates, a substrate containing tetramethylbenzidine (TMB) and 0.05% hydrogen peroxide was added and incubated at room temperature for 10 min. The enzymatic coloring reaction was stopped by adding 1.25 M sulfuric acid. Extinctions were measured with an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, Vermont, USA) at a wavelength of 450 nm. Levels of IgY and IgM antibodies binding KLH were expressed as titers being the log2 values of the dilutions that gave an extinction closest to 50 per cent of Emax, where Emax represents the highest mean extinction of a standard positive (pooled) plasma present on every microtiter plate [32].

Determination of Meat Cholesterol

Meat cholesterol analysis has been described in an earlier study by Kumalasari et al.[27]. In brief, meat cholesterols were determined using the enzymatic calorimetry test method Cholesterol Oxidase Phenylperoxidase Aminophenazone (CHOD-PAP). The meat sample was ground and weighed 1 g, then put into an empty tube. Three ml of ether solution was added, left for 24 hours, and homogenized. Centrifuge at 3000 rpm for 15 min. The supernatant was transferred into an Eppendorf tube. The blank cuvette was filled with 1000 µL of reagent and 10 µL of aquadest. The standard cuvette is filled with 1000 µL of reagent and 10 µL of standard. The assay cuvette (sample) is filled with 1000 µL of reagent and specimen as much as 10 µL. The blank absorbance value is read using a spectrophotometer wavelength of 500 nm [32].

Statistical Analysis

Data were analyzed by using one-way analysis of variance (ANOVA) in a general linear model (proc GLM) of SAS version 9.2 (Cary, NC, USA) to determine effects of different probiotics culture media at different time points. Body weight was analyzed on a weekly basis for 4 weeks (week 0, week 1, week 2, week 3, and week 4). Body weight gain, average daily gain, feed intake, and feed conversion ratio (FCR) were analyzed on a biweekly basis, starting on week 0 of the experimental period (week 0-2, week 2-4) and week 0-4). Moreover, blood parameters, natural antibody titers were also analyzed bi-weekly, starting from week 0 of the experimental period (week 0, week 2, and week 4). Carcass percentage, meat cholesterol, abdominal

fat, and weight of bursa Fabricius were analyzed at week 4. The effects of different probiotics culture media were considered significant at P≤0.05. The analyses were then continued with Duncan's multiple range test as multiple comparisons. The data were expressed as least square means (LSM) of the respective parameter with pooled standard error of means (SEM).

Data from blood parameters, natural antibodies, and lipid metabolism were analyzed using the MIXED procedure (PROC MIXED) in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). A repeated-measures mixed model was used to assess the fixed effects of treatment (T), time (week, W), and their interaction (TxW), with the individual replicate (pen) specified as a random effect. Covariance structures (autoregressive 1) were tested and selected based on the lowest Akaike Information Criterion (AIC). Least square means (LSM) were compared using Duncan's multiple range test, and significance was declared at P≤0.05.

Results

Probiotic Characteristic

Probiotic characteristic such as pH, lactic acid levels, total lactic acid bacteria and the total bacteria was not significant difference among treatments *(Table 3)*. However, MM had higher total lactic acid bacteria compared with CM and SM.

Performance and Carcass Yield

During the experiment, the mortality of birds was not exceeding 4% of the total population, therefore it was neglected in the statistical analysis. At the day 1, the probiotics in different culture media treatments were not yet administered, therefore it can be considered as default status *(Table 4)*. However, birds were already grouped in day 1. In day 1, there was a statistically significant difference in body weight, i.e. a lower body weight in the SM-fed group compared to other groups (P<0.0001). Though statistically significant, the actual mean difference in body weight was not exceeding 1.5 g, therefore it can still be considered homogenous.

Current study showed that body weight gain, average daily gain, feed intake, and final body weight were significantly higher in bird fed SM treatment compared with birds fed CT group *(Table 3)* especially from week 2 to week 4. The final body weight of birds in SM fed group was significantly higher compared to other groups, especially compared to the CM fed group (P<0.05). This is in line with the feed intake in the SM fed group which is significantly higher compared to other treatment groups. The lower FCR in the SM fed group also reflected this.

Blood Parameters and Immune Response

In the week 0, there was no significant difference in plasma metabolites, and plasma Nabs except in titers of IgM. This study showed there was significant difference in plasma metabolites after the administration of probiotics dietary treatments. *Table 5* showed that birds fed MM treatments had higher plasma total protein (P<0.01) compared with other treatments. Birds fed CT treatments had higher plasma triglycerides (P<0.1) compared with other treatments. Birds fed MM treatments had higher plasma total protein (P<0.001) compared with other treatments. There was significant difference in plasma metabolites between weeks *(Table 5)*.

There was no significant difference in plasma Nabs except in titers of IgM *(Table 6)*. At week 2, with respect to levels of plasma Nabs, the IgM anti-KLH titer tended to be higher in the SM fed group compared to the CM fed group. The plasma IgM titer decreased in the CT, CM, and SM fed birds from week 0 to 2, while, in contrast, the plasma IgM titer in MM fed birds increased in the same period. SM fed birds had higher carcass percentages with the lower abdominal fat percentage and lower cholesterol in plasma and meat compared with other treatments. Triglyceride, total protein, and albumin levels in plasma of SM fed birds were significantly lower compared to the other treatment groups (P<0.01). Moreover, SM fed birds had lower levels of IgY and IgM antibodies binding keyhole limpet hemocyanin (KLH) compared with the control group.

In week 4, plasma cholesterol levels in the SM and MM-fed groups were significantly lower compared with CT and CM groups (P<0.01), suggesting a low level of fat

Table 3. *Physicochemical properties and viable microbial counts of probiotic treatment in this study*

Item	Treatment			SEM	P-value
	CM	SM	MM		
pH	3.97	3.78	4.03	0.68	0.31
Lactic acid level (%)	0.68	0.74	0.73	0.09	0.19
Total lactic acid bacteria (CFU/g)	4.51×10^3	3.85×10^3	7.09×10^2	n.a[2]	n.a[2]
Total bacteria (CFU/g)	1.27×10^3	1.70×10^3	1.28×10^3	n.a[2]	n.a[2]

CM: *basal ration + 2% of probiotics cultured in cow milk (100%) medium;* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean emulsion (50%) media;* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean emulsion (25%) media; [2]n.a: not applicable*

Table 4. *Effects of different lactic acid bacteria culture media on performance*

Treatment	Body Weight Gain (g/head)			Average Daily Gain (g/head/day)			Feed Intake (g/head)			Feed Conversion Ratio			Final BW (g)	Mortality (%)
	0-2 wk	2-4 wk	0-4 wk	0-2 wk	2-4 wk	0-4 wk	0-2 wk	2-4 wk	0-4 wk	0-2 wk	2-4 wk	0-4 wk		
CT	325.6	922.3^{b}	1247.9^{b}	23.26	65.9^{b}	44.6^{b}	428.2	1258.1	1686.3^{b}	1.32	1.37^{b}	1.36^{b}	1294.4^{a}	3.63
CM	328.0	901.0^{c}	1229^{bc}	23.43	64.4^{c}	43.9^{bc}	427.6	1422.5	1850.1^{a}	1.30	1.58^{a}	1.51^{a}	1275.4^{bc}	0
SM	328.5	959.0^{a}	1287.4^{a}	23.46	68.5^{a}	46.0^{a}	431.7	1461.4	1893.0^{a}	1.31	1.52^{a}	1.47^{a}	1332.4^{a}	1.82
MM	327.8	889.6^{c}	1217.4^{c}	23.41	63.5^{c}	43.5^{c}	426.7	1395.4	1822.1^{a}	1.30	1.57^{a}	1.5^{a}	1263.3^{c}	0
SEM	1.62	4.51	4.91	0.12	0.32	0.18	1.76	20.97	20.58	0.003	0.024	0.017	4.5	-
P-value	0.93	<.001	<.001	0.93	<.001	<.001	0.77	0.0027	0.0018	0.2368	0.0048	0.0072	<.001	-

CT: *control (no probiotics supplementation);* ***CM:*** *basal ration + 2% of probiotics cultured in cow milk (100%);* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean milk (50%);* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean milk (25%); ** P<0.01; *** P<0.0001;* ^{a,b,c}*Means with different superscripts within the same column are different in accordance with respective significance levels*

Table 5. *Effects of different lactic acid bacteria culture media on blood parameters*

Item	Treatment				SEM	Week			SEM	P-values	
	CT	CM	SM	MM		0	2	4		T	W
Total protein (g/dL)	2.53^{b}	2.55^{c}	2.60^{b}	3.13^{a}	0.12	1.2717^{c}	2.9642^{b}	3.8734^{a}	0.10	0.0011	<.001
Albumin (g/dL)	5.62	5.26	4.95	5.39	0.18	4.24^{c}	5.77^{b}	5.91^{a}	0.15	0.0775	<.001
Blood urea nitrogen (mg/dL)	4.47	4.62	4.27	5.52	0.43	4.75^{b}	3.91^{c}	5.50^{a}	0.36	0.1826	0.0036
Triglyceride (mg/dL)	151.94^{a}	130.1^{b}	110.49^{d}	111.63^{c}	8.93	147.93^{c}	133.75^{b}	96.4385^{a}	7.53	0.0032	<.001

CT: *control (no probiotics supplementation);* ***CM:*** *basal ration + 2% of probiotics cultured in cow milk (100%) medium;* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean emulsion (50%) media;* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean emulsion (25%) media; ** P<0.01; *** P<0.0001.* ***T:*** *fixed effect of treatment;* ***W:*** *fixed effect of week (time);* ^{a,b,c} *Means with different superscripts within the same row are different in accordance with respective significance levels*

Table 6. *Effects of different lactic acid bacteria culture media on natural antibodies binding Keyhole Limpet Hemocyanin (KLH)*

Item	Treatment (T)				SEM	Week			SEM	P-values	
	CT	CM	SM	MM		0	2	4		T	W
IgY titer	5.34^{a}	3.83^{b}	3.33^{c}	3.13^{a}	0.34	4.41	1.94	5.37	0.28	<.001	0.1072
IgM titer	5.40^{a}	4.39^{b}	4.57^{b}	3.46^{c}	0.36	4.80	3.72	4.85	0.29	0.0025	0.325
Bursa of Fabricius weight (g)	1.34	1.40	1.12	0.96	0.09				0.09	0.22	

CT: *control (no probiotics supplementation);* ***CM:*** *basal ration + 2% of probiotics cultured in cow milk (100%) medium;* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean emulsion (50%) media;* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean emulsion (25%) media; * P<0.05; ** P<0.01; *** P<0.0001;* ***T:*** *fixed effect of treatment;* ***W:*** *fixed effect of week (time);* ^{a,b,c} *Means with different superscripts within the same row are different in accordance with respective significance levels*

Table 7. *Effects of different lactic acid bacteria culture media on lipid metabolism*

Item	Treatment				SEM	Week			SEM	P-values		
	CT	CM	SM	MM		0	2	4		T	W	T×W
Plasma cholesterol (mg/dL)	186.23	192.12	184.26	186.9	4.70	280.08^{a}	139.55^{c}	142.5^{b}	3.96	0.68	<.001	0.001
Meat cholesterol	36.19^{a}	31.51^{b}	27.97^{b}	29.07^{b}	1.05	n.a	n.a			<.001	n.a	n.a
Carcass percentage (%)	67.32^{b}	67.56^{b}	68.43^{a}	63.82^{c}	0.76					<.001		
Abdominal fat (g)	1.42^{ab}	1.59^{a}	1.11^{b}	1.10^{b}	0.12					0.01		

CT: *control (no probiotics supplementation);* ***CM:*** *basal ration + 2% of probiotics cultured in cow milk (100%) medium;* ***SM:*** *basal ration + 2% of probiotics cultured in cow milk (50%) and soybean emulsion (50%) media;* ***MM:*** *basal ration + 2% of probiotics cultured in cow milk (50%), soybean emulsion (25%), and mungbean emulsion (25%) media; * P<0.05; ** P<0.01; *** P<0.0001;* ***T:*** *fixed effect of treatment;* ***W:*** *fixed effect of week (time). n.a: not applicable;* ^{a,b,c} *Means with different superscripts within the same row are different in accordance with respective significance levels*

mobilization. Levels of total protein were higher in the CT-fed group compared to other treatment groups (P<0.01), suggesting a high protein transport. However, plasma albumin levels were lower in the CT-fed group, but insignificant. This suggestion is supported by the fact that levels of IgY-binding KLH in the CT-fed group were significantly higher compared to other treatment groups. (P<0.001). Titers of natural antibodies IgY and IgM binding KLH in SM-fed birds were higher than CM and MM-fed birds (P<0.05).

Lipid Metabolism

In week 4, plasma triglyceride and cholesterol levels in the SM and MM-fed groups were significantly lower compared with CT and CM groups (P<0.01), suggesting a low level of fat mobilization. Birds fed SM had lower cholesterol levels in meat *(Table 7)*. In terms of abdominal fat, a lower percentage was found in SM and MM fed groups, compared to the CT and CM fed group (P<0.01). The SM fed group had a higher carcass percentage compared to other treatment groups (P<0.01), suggesting a higher inedible parts percentage in MM fed birds.

Discussion

The week 0 data depict the actual situation of commercial poultry practices in Indonesia, showing extended levels of variation in day-old chick conditions.

While pH, lactic acid levels, and total bacterial counts were similar across treatments, the MM culture medium supported significantly higher LAB growth. This may be attributed to the specific nutrient composition of the MM formulation, which combines cow milk, soybean emulsion, and mung bean emulsion [33,34]. These components may create a synergistic effect, enhancing microbial proliferation. Although the pH values in our study (3.78-4.03) were below the optimal bacteriocin production range of 6.2-8.5 [35], the MM medium's relatively higher pH (4.03) may still offer a more hospitable environment for LAB survival. Prior findings have suggested that cow milk provides readily available lactose for LAB, while soybean and mung bean components contribute prebiotic fibers and bioactive compounds that may further promote LAB activity [34].

The differing carbohydrate profiles of cow milk, soybean, and mung bean may explain the variations in LAB viability. Cow milk offers simple sugars like lactose, which LAB strains such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* readily utilize [36]. In contrast, soybean and mung bean provide oligosaccharides such as raffinose and stachyose, substrates more selectively metabolized by *Bifidobacterium bifidum* [37].

Current study showed a significant difference on growth performance, carcass yield, abdominal fat including other lipid metabolism among treatments especially at week 4. Week 4 in the present study can be regarded as a final time point that reflects the condition of birds to be harvested in commercial poultry circumstances. The final body weight of birds in SM fed group was significantly higher compared to other groups, especially compared to the CM fed group. This is in line with the feed intake in the SM fed group which is significantly higher compared to other treatment groups. The lower feed FCR in the SM fed group also reflected this. The carcass percentage was also higher in the SM fed group compared to other treatment group, but not significant. In terms of lipid metabolism including plasma triglyceride, levels of cholesterol in plasma and in meat and abdominal fat, a lower percentage was found in SM fed groups compared to the CT and CM fed group (P<0.01).

The SM fed birds not only had higher final body weight, but also higher body weight gain and feed intake compared with the other treatments. In addition, SM fed birds had better FCR compared with the control group. The MM fed group in week 2 had a lower carcass percentage compared to other treatment groups (P<0.01), suggesting a higher inedible parts percentage in MM fed birds.

With respect to blood parameters, significantly higher plasma triglycerides levels in the CT fed were found compared to other treatment groups (P<0.0001), suggesting a higher adipose fat transport and glucose transfer in the CT fed group at week 2 compared to the other treatment groups. Previous studies have shown that probiotic supplementation in laying hen feed has a significant effect in reducing blood triglyceride levels [38]. Meanwhile, other studies have shown that probiotic supplementation in layer feed tends to reduce blood triglyceride levels although not significantly different [33]. In this study, we observed that supplementation of probiotic with different culture media in the diet of broiler also reduce plasma triglyceride levels. The decrease in plasma triglyceride levels could be due to the supplementation of probiotic, which affects the fatty acid synthesis process in the body of the hens. According to previous studies, microbiota in probiotics can effectively reduce the activity of acetyl-CoA carboxylase (ACC), which is an enzyme involved in the rate of fatty acid synthesis. Less secretion of ACC results in less formation of fatty acids and decreased fatty acid formation lowers blood triglyceride levels [38]. Thus, decreased blood triglyceride levels lead to decreased triglyceride levels synthesized in the liver. In addition, according to Mayasari and Adriani [38], probiotics can also assimilate cholesterol, leading to impaired micelle formation. Lower micelle formation decreases the uptake of lipids in the intestinal lumen, ultimately reducing the number of circulating triglycerides in the blood. This

will lead to a decrease in the uptake of lipid profile in the blood. The change in lipid profile distribution especially cholesterol may be due to increased biosynthesis and accumulation in the liver. A previous study showed that probiotic supplementation decreased plasma cholesterol levels in broilers [39]. Supplementation of lactic acid bacteria in diet or water was related to low cholesterol levels in the plasma which may be due to LAB causing a decrease in gallbladder acid secretion. Low secretion of gallbladder acid decreased the ability of fat digestion and therefore decreased lipid levels in the blood [40]. In addition, bioactive compounds both in soybean (glycinin, lecithin) and mung bean (phytosterol) were shown to decrease cholesterol in plasma [41]. Moreover, soybean and mung bean contain resistant starch which increased the production of cecal short-chain fatty acid (SCFA, eg. Butyrate), and elevated fecal neutral sterol excretion, thereby reducing the serum total cholesterol level [40]. Regarding the blood parameters, the levels of total protein were significantly higher in the MM-fed group compared to other treatment groups ($P<0.01$), suggesting a high protein transport. However, plasma albumin levels were lower in the CT-fed group, but insignificant. A critical factor in the protein depositing into meat is the levels of protein and albumin. The high level of total protein and low level of albumin might suggest that the proportion of the major protein type transported is not albumin, but globulin. This lower proportion of albumin compared to globulin might suggest a high mobilization in immune system build-up, especially for IgY formation. This suggestion is supported by the fact that levels of IgY-binding KLH in the CT-fed group were significantly higher compared to other treatment groups. ($P<0.001$). Titers of natural antibodies IgY and IgM binding KLH in SM-fed birds were higher than CM and MM-fed birds ($P<0.05$).

This lower proportion of albumin compared to globulin might suggest a high mobilization in immune system build-up, especially for IgY formation. The suggestion is supported by the fact that levels of IgY-binding KLH in the CT-fed group were significantly higher compared to other treatment groups. ($P<0.001$). Titers of natural antibodies IgY and IgM binding KLH in SM-fed birds were higher than CM and MM-fed birds ($P<0.05$). This aligns with a previous study that dietary supplementation of fermented soybean meal could increase immune responses in broilers [42]. Probiotics effectively remove anti-nutritional elements and increase the nutritional value by fermenting soybean meal, which results in the production of numerous enzymes [43], therefore the performance is enhanced as well as the immune response.

With respect to levels of plasma Nabs, the IgM anti-KLH titer tended to be higher in the SM fed group compared to the CM fed group. The plasma IgM titer decreased in the CT, CM, and SM fed birds from week 0 to 2, while, in contrast, the plasma IgM titer in MM fed birds increased in the same period. The observed elevation of IgM might be related to activation of the immune system [41]. In terms of IgY, a decreased level was observed in all treatments from week 0 to week 2. A previous study suggested that chicks' IgY was maternally derived until at least 8 days of life [44], implying that the decreased level of IgY in week 2 could reflect the remnants of this maternal derivation. Regarding treatment effects, MM-fed birds exhibited a significantly lower IgY level in week 2 compared to CT-fed birds ($P<0.05$), indicating an accelerated breakdown or usage of the maternal antibody remnants. Nabs are immunoglobulins that are present in individuals who have had no known exposure to antigens in the past [45]. Recent studies have suggested that the levels of Nabs in an individual animal may serve as a reliable indicator of their immune competence [26], with higher Nabs levels in poultry indicating a stronger and more efficient immune response [46]. The availability of nutrients in broilers may have been increased by microbial fermentation since it was shown that fermentation reduced antinutritional factors in soybean and boost small-size peptides [47].

Although the diets were not strictly isoenergetic, the linear trends observed across treatments for several parameters (e.g., growth performance and lipid profiles) suggest that the positive effects were not solely due to variations in nutrient content. Rather, the results likely reflect a combined influence of probiotic activity and nutritional composition, with the probiotic effect becoming more evident as the complexity of the culture media increased.

In conclusion, different culture media of lactic acid bacteria (cow's milk, soybean milk and mung bean milk) affected performance, plasma metabolites, and immune status. We tried to determine culture media that support growth and activity of LAB as alternative for cow milk. The best result was obtained with probiotic in a combination of cow's milk and soybean milk. Based on the results of this research, birds fed probiotics cultured in cow's milk and soybean milk showed better growth performance, an increased carcass percentage, lower abdominal fat, lower levels of plasma cholesterol, and higher titers of IgY binding KLH representing natural antibodies. Thus, supplementation of probiotics with combination of specific culture media can be effectively used to ensure favorable results, not only for growth performance and immune response but also to replace antibiotics.

However, this study was limited by its relatively short trial duration and the absence of molecular identification of microbial changes in the gut. Additionally, the strain-specific effects of probiotics were not distinguished. Future studies are encouraged to investigate the underlying microbiota shifts using metagenomic tools,

explore longer-term impacts through the full production cycle, and assess cost-effectiveness at scale for commercial application.

Declarations

Availability of Data and Materials: The authors declare that data supporting the study findings are also available from the corresponding author (N. Mayasari) on reasonable request.

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