Relationship Between Growth Performance and Metabolite Profile of Broiler Chickens Supplemented with Probiotics *Bacillus coagulans* and *Lactobacillus plantarum*

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ABSTRAK

Pradhika EI, Astuti RI, Meryandini A. 2025. Hubungan performa tubuh dan profil metabolit pada ayam broiler dengan suplementasi probiotik *Bacillus coagulans* and *Lactobacillus plantarum*. JITV 30(2):115-125. DOI:http://dx.doi.org/10.14334/jitv.v30i1.3453.

Suplementasi probiotik merupakan alternatif dari Antibiotic Growth Promotor. Probiotik L. plantarum dan B. coagulans diketahui dapat meningkatkan performa pertumbuhan ayam broiler. Informasi mengenai hasil metabolit kedua probiotik dengan inangnya masih terbatas. Penelitian ini bertujuan untuk mengidentifikasi metabolit pembeda antara Bacillus coagulans dan Lactobacillus plantarum dan metabolit yang berhubungan dengan peningkatan performa pertumbuhan ayam broiler dengan hasil suplementasi probiotik . Sebanyak 120 ekor Ayam Broiler unsexRoss 308 diberikan pakan perlakuan dengan Kontrol Negatif (NC), L. plantarum (LP), B. coagulans (BC), dan B. coagulans dicampur dengan L. plantarum (BCLP). Parameter kinerja pertumbuhan yang dievaluasi adalah rata-rata bobot badan (average Body Weight/ avg BW), konversi pakan (adjustment Feed Conversion Ratio/adjFCR), konsumsi pakan kumulatif(cumulative Feed Intake/cumFI) dan faktor efisiensi performa (Performance Efficiency Factor/PEF). Analisis metabolik dilakukan dengan metode pemprofilan metabolit tidak tertarget pada sampel sekum yang terdiri dari analisis senyawa spektrum luas dan analisis senyawa volatil. Hasil penelitian menunjukkan bahwa kinerja pertumbuhan (avg BW) yang berbeda nyata ($p \leq 0,05$). Sedangkan parameter lainnya yaitu, adjFCR, cumFI, dan PEF, tidak memberikan perbedaan yang signifikan ($P > 0,05$). Metabolit pembeda yang penting antar perlakuan adalah asam asetat, asam laktat, asam butanoat, 1-oktadekanol, dan asam palmitat. Metabolit yang dapat dinyatakan sebagai metabolit pembeda antara LP dan BC adalah asam asetat, asam laktat, dan asam butanoat. Sedangkan metabolit yang dapat dinyatakan sebagai metabolit pembeda adalah asam laktat sebagai pembeda kinerja avgBW yang baik dan 1-oktadekanol dan asam palmitat sebagai pembeda tanpa suplementasi probiotik. Kesimpulan dari studi ini adalah asam asetat, asam laktat dan asam butanoat adalah metabolit pembeda antara probiotik B. coagulans dan L. plantarum dan asam laktat sebagai metabolit pembeda kinerja yang baik.

Kata Kunci: Ayam Broiler, Metabolit Pembeda, Metabolit, Performa, Probiotik

ABSTRACT

Pradhika EI, Astuti RI, Meryandini A. 2025. Relationship of growth relationship between growth performance and metabolite profile of broiler chickens supplemented with probiotics *Bacillus coagulans* and *Lactobacillus plantarum*. JITV 30(2): 115-125. DOI:http://dx.doi.org/10.14334/jitv.v30i1.3453.

Probiotic supplementation is an alternative to Antibiotics Growth Promotor. The probiotics L. plantarum and B. coagulans are known to improve the growth performance of broiler chickens. Information regarding the metabolite results of these two probiotics with their hosts is still limited. This study aims to identify differentiating metabolites between Bacillus coagulans and Lactobacillus plantarum and metabolites associated with enhanced growth performance in chickens supplemented with probiotics. A total of 120 unsexed Ross 308 Broilers were given a treated diet with Negative Control (NC), L. plantarum (LP), B. coagulans (BC), and B. coagulans mixed with L. plantarum (BCLP). The growth performance parameter evaluated was the average Body Weight (average BW), adjustment Feed Conversion Ratio (adjFCR), cumulative Feed Intake (cumFI), and Performance Efficiency Factor (PEF). Metabolomic analysis was carried out using the untargeted metabolite profiling method on cecum samples, consisting of broad-spectrum and volatile compound analyses. The study shows that analysis of differences in growth performance resulted in only the avgBW parameter being significantly different ($P \leq 0.05$). Meanwhile, other performance parameters, adjFCR, cumFI, and PEF, do not provide any significant difference ($P > 0.05$). The important differentiating metabolites between treatments are acetic acid, lactic acid, butanoic acid, 1-octadecanol, and palmitic acid. Metabolites that can be stated as differentiating metabolites between LP and BC are acetic acid, lactic acid, and butanoic acid. Meanwhile, metabolites that can be declared differentiating metabolites are lactic acid as a differentiator for good avgBW performance and 1-octadecanol and palmitic acid as differentiators without probiotic supplementation. It can be concluded that

acetic acid, lactic acid, and butanoic acid are the metabolites that differentiate the probiotics *B. coagulans* and *L. plantarum* and lactic acid as a differentiating metabolite of good performance.

Key Words: Broiler Chicken, Differentiating Metabolites, Metabolite, Performance, Probiotics

INTRODUCTION

The chicken farming industry continues to develop to meet the increasing need for animal protein. One important factor in achieving optimal growth and health of chickens is using feed enriched with AGP (Antibiotic Growth Promoter). The prohibition of AGP dramatically influences the productivity of broiler chickens in Indonesia as an implementation of Law (UU) Number 41 of 2014 concerning Amendments to Law Number 18 of 2009 concerning Livestock and Animal Health concerning the ban on using antibiotics and certain hormones as feed additives; this encourages researchers to develop safe and effective AGP alternatives. One solution to this problem, called AGP replacer, is probiotic supplementation. Probiotics have been widely used in the feed industry today as AGP replacers. Some of the advantages of using probiotics in the digestive tract are stimulating beneficial microbes, preventing pathogen colonization by competition for attachment to the epithelium, reducing pH conditions, producing organic acids, forming compounds with systemic effects, and modulating the immune system (Abd El-Hack et al. 2020; Tran et al. 2022).

One of the LAB (Lactic Acid Bacteria) based probiotics is the *L. plantarum* strain. Feed supplemented with *L. plantarum* strain B1 was shown to reduce the number of *E. coli*, increase other LAB bacteria, produce SCFA (Short Chain Fatty Acid), and improve broiler performance (Peng et al. 2016). One type of SFB (Spore Former Bacteria) used as probiotics is *B. coagulans* (Gu et al. 2015). *B. coagulans* strain R11 was shown to prevent oxidative damage and reduce the abundance of pathogens such as *E. coli*, *P. aeruginosa*, and Salmonella (Xing et al. 2020). One approach to studying the efficacy of probiotics on the health of their hosts is through the metabolomics approach. Metabolite profiling of untargeted compounds allows for identifying compounds that undergo significant concentration changes under different treatment conditions (Frainay and Jourdan 2017).

Liu et al. (2020) explained that the metabolite results from probiotics could generally be lactic acid, hydrogen peroxide, secreted proteins (extracellular proteins), organic acids, indole, bacteriocins, and antimicrobial peptides. Wang et al. (2021) provide an overview of the characteristics of LAB metabolites as probiotic functions, including being able to produce short-chain fatty acids, amines, bacteriocins, vitamins and exopolysaccharides. According to Elshaghabee et al. (2017), Bacillus spp. stimulate the immune system and produce several antimicrobial substances, e.g., bacteriocins like inhibitory substances and antibiotics.

Probiotics based on *L. plantarum* and *B. coagulans* improve broiler growth performance (Khajeh Bami et al. 2020; Peng et al. 2016). However, this characteristic is unclear in identifying metabolite compounds that distinguish the two types of probiotics. The metabolomic analysis provides an overview of the diversity of metabolite compounds from probiotics. Therefore, it is necessary to know the profile of metabolite diversity between the two probiotics, which is associated with growth performance. This study aimed to identify the differences in metabolite profiles between *B. coagulans* and *L. plantarum* and to determine the metabolites that distinguish good growth performance.

MATERIALS AND METHODS

This research was conducted at the research farm (AME House/Digestibility Assay Unit, House 7, Room A & B, closed house) of PT Nugen Bioscience Indonesia, Malingping, Banten and applied chemistry department laboratory of PT Nugen Bioscience Indonesia, Ancol, North Jakarta. The Animal Ethics Committee School of Veterinary Medicine and Biomedical Science IPB University approved the experiment with approval number 070/KEH/SKE/VII/2023.

Treatment, experimental design and poultry management

One hundred and twenty DOC Broiler (46±0.1g) Ross 308 grade A3 unsex (PT Charoen Pokphand Jaya Farm, Tangerang, Indonesia) were randomly divided into four dietary treatments: LP (*L. plantarum*), BC (*B. coagulans*), BCLP (*B. coagulans* and *L. plantarum*) and NC (no probiotics) with 6 replicates per treatment and 5 bird per replicates. Twenty-four cages were arranged on racks randomly divided into two rooms (rooms A and B). Each room contained 12 cages with three replications of each treatment. Each cage (0.61×0.61×0.39 m, 0.145 m^3) consists of 2 nipple drinkers/cage, 1 bell drinker/cage, 1 feeder/cage, and 1 lamp/cage. Each room (8×5×2.5 m, 100 m^3) contains 4 cage racks/room, 1 fan unit/room, and 1 water tank unit/room. Cages were cleaned with disinfectant [peracetic acid-hydrogen peroxide-acetic acid (Cid 2000, PT SHS International, Jakarta, Indonesia)].

Rooms were fumigated (75 g peroxyacetic acid (Forcent Fumigant, PT Indovetraco Makmur Abadi, Jakarta, Indonesia) added to 150 ml formaldehyde 37 % (Formac, PT Indovetraco Makmur Abadi, Jakarta, Indonesia) for an area of 5 m^3) before use to prevent contamination. DOCs were weighed before being put into the cages. Feeding was done using an ad libitum feeding system according to the treatment. In the starter/brooding phase (0-10 days), DOC was fed with S10 feed, and in the grower and finisher phases (11-30 days), they were fed with S11 feed, which has been supplemented with probiotic products according to the treatment. The nutritional content of the feed can be seen in Table 1. Room temperature was maintained according to Ross 308 guidelines (Aviagen, 2018) by adding a heat source lamp in the brooding phase, adjusting the frequency of ventilation opening, and setting the fan switch. Chicken performance was determined by measuring avgBW (average Body Weight at 10, 21, and 30 days), cumFI (cumulative Feed Intake), adjFCR (adjusted Feed Conversion Ratio), PEF (Performance Efficiency Factor), and mortality parameters calculated according to Ross 308 Aviagen (2018) and Martínez & Valdivié (2021) standard guidelines which can be seen in the Table 2. All chickens were then slaughtered, and cecum content samples were aseptically removed from the chickens using scissors and tweezers and placed into labeled sterile tubes. Samples were frozen with dry ice in an ice box and then stored in an ultra-low temperature freezer (Kaltis 390, Taipei, Taiwan) at -80°C, according to Zhou et al. (2021).

Feed preparation

Probiotic products consist of *L. plantarum* N1A1 or *B. coagulans* BR04 mixed in a carrier (corn starch and $CaCO_3$) (PT Nugen Bioscience Indonesia, Jakarta, Indonesia) with concentration >10^6 CFU/g. Starter (S10) and grower-finisher (S11) phase feeds (PT Charoen Pokphand Indonesia, Balaraja, Indonesia) were each mixed with 1.5 % probiotic product to produce a minimum concentration of 10^8 CFU/kg feed as recommended by Wang et al. (2021). Feed is mixed with a mini-feed mixer for 15 minutes at room temperature. The NC treatment was supplemented with products without probiotics (only carriers).

Metabolomic analysis

. The extraction and derivatization step for metabolomic analysis of untargeted broad-spectrum compounds was adopted from Fiehn (2016) of standard mix QC. Acetonitrile:Isopropanol: Water (AIW) solution (Merck, Darmstadt, Germany) with a ratio of 3:3:2 was purged with N_2 gas from gas generator (Proton N341M, Proton OnSite, USA) for 5 min and then cooled at -20 °C. 25-30 mg of cecum content sample was weighed with analytical balance (Precisa XB 220A, Dietikon, Switzerland) into a 2 ml microtube, and 1 ml of AIW was added. The microtube was mixed with a vortex (Heidolph REAX control, Schwabach) for 10 s followed by one h sonication (Elmasonic P300H, Singen, Germany) at 35°C and then centrifuged (Biofuge Fresco Sorvall, Thermo Fisher Scientific, Waltham, USA) at 13,000 ×*g* for 2 min. 450 µl of supernatant was separated into a new microtube and concentrated with a vacuum concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany) for 2 hours at 45°C. MeOX solution was prepared by mixing 20 mg methoxyamine HCl (Sigma Aldrich, Massachusetts, USA) and 1 ml pyridine (Merck, Darmstadt, Germany), then sonicated for 15 min at 60 °C. The concentrated microtube was added with 50 µl MeOX, followed by 1.5 hours of sonication at 30°C. Then 100 µl of MSTFA (N-methyl-N-(trimethylsilyl)-trifuoroacetamide, Merck, Darmstadt, Germany) was added and sonicated for one h at 37°C and then centrifuged at 13,000 ×*g* for 10 min at 18°C. The supernatant obtained was then transferred to a vial insert and placed into a GC vial. The samples in the vials were then randomly arranged and analyzed using a gas chromatography system with specifications according to Jain et al. (2019) below. GC system: Agilent 7000C Triple Quadrupole GC/MS System (Agilent, Santa Clara, USA); column: HP-5MS Ultra

Table 1. Nutritional content of S10 and S11 feed

Parameter	S10 feed	S11 feed
Moisture (%)	10.59	10.79
Fat (%)	5.56	6.04
Fiber (%)	4.94	6.32
Protein (%)	20.22	19.31
Ash (%)	5.44	5.39
ME (kkal/kg)	3155	3122

ME= Metabolism Energy

Table 2. Calculation formula for performance parameter

Parameter	Formula
avgBW (g/bird)	total bird weight/number of birds.
cumFI (g/bird)	Average daily Feed Intake (avgDFI) × number of birds × number of days
adjFCR	actFCR + (target body weight – actual body weight / 4500 g).
actFCR	total feed consumed / total bird weight
PEF	livability × bird weight (kg) / age (days) × FCR
livability (%)	The final number of birds/initial number of birds × 100
mortality (%)	total death or culling/number of birds × 100

Inert (30 m×0.25 mm×0.25 µm); gas: He (2.25 ml/min); injection volume: 1 µl; delay: 4 min; inlet: splitless, 250°C, 14.7 psi; oven: 75°C, 280°C (4°C/min, 1.56 min); detector: MS, source: 230°C, 40-600 m/z, scan time: 0.2 s.

Extraction and derivatization methods in metabolomics analysis for volatile targeted compounds were adopted from Hsu et al. (2019). Partially frozen samples stored at -80°C were freeze-dried with a freeze dryer for 24 hours. Samples that were not analyzed immediately could be stored again at -80°C. A dry sample of 0.02 g was weighed in a microtube, and 1 ml of 0.5% phosphoric acid (Merck, Darmstadt, Germany) was added. The sample was vortexed for 30 s and then centrifuged at 3,000×*g* for 10 min. 60 µl of supernatant was removed, and 240 µl of 0.5% phosphoric acid and 300 µl of butanol (Merck, Darmstadt, Germany) were added. The sample was vortexed for 30 s, shaken for 5 min, followed by sonication for 5 min. Then, the microtube was centrifuged at 3,000 ×*g* for 10 min. 180 µl of supernatant (organic layer) was transferred to a vial insert, and 20 µl of butanol was added. The samples in the vials were then randomly arranged and analyzed using a gas chromatography system according to the following specifications. GC system: Agilent 7000C Triple Quadrupole GC/MS System (Agilent, Santa Clara, USA); column: DB-WAXms (30 m×0.25 mm×0.25 µm); gas: He (2.25 ml/min); injection volume: 1 µl; delay: 4 min; inlet: splitless, 250°C, 14.7 psi; oven: 70°C, 170°C (10°C/min, 0 min), 240°C (25 °C/min, 15 min); detector: MS, source: 230°C, 40-550 m/z, scan time: 0.2 sec. Solvent blank, reagent (derivatization) blank, and method blank were selected as quality control for each batch analysis (Fiehn 2016; Broadhurst et al. 2018; Eurachem 2019).

Data analysis

Raw growth performance data were processed, and the significance of performance was determined by statistical analysis on Minitab® 16.1.1.1 (Minitab Ltd, Pennsylvania, USA). The statistical analysis stages performed were (1) outlier identification (NIQR box-plot), (2) assumption checking (data normality test: Shapiro-Wilk test, homogeneity or homoscedasticity of data: Bartlett test), (3) omnibus test (parametric test: ANOVA or non-parametric test: Kruskal-Wallis), and (4) post hoc test (parametric test: Tukey test or non-parametric test: Dunn test) (Granato et al. 2014). Non-parametric tests are performed for samples <15 data.

Chromatogram data from the metabolomic analysis was processed using Masshunter Qualitative Analysis B.07.00 software (Agilent, Santa Clara, USA). Chromatogram peaks with a minimum height of 10^5 mAU (mili Absorbance Unit) were identified from the TIC (Total Ion Chromatogram), and then the deconvolution process was performed. The detected peaks were then matched to the National Institute of Standards and Technology (NIST) database with a similarity score of at least 80 %. Raw data in peak intensity height, RT (Retention Time), and compound name were processed in MS Excel by adopting the procedure from Fiehn (2016). Curation data from the analysis of broad-spectrum and volatile compounds were combined into one, then outlier identification, compound name filtering and box-plot generation using MS Excel. Multivariate analysis and compound categorization were performed with MetaboAnalyst 5.0 (Wishart Research Group, Alberta, Canada). The compounds obtained were grouped by class using the 'Enrichment Analysis' feature. All compounds identified by NIST from the two metabolomic analyses were confirmed by (the Human Metabolome Database) HMDB library-based matching. Compounds identified but not matched and indicated not to be metabolites were excluded from further analysis. The proportion (p) of data was determined by calculating the number of metabolites that appeared (x) per number of samples (n). Identified metabolites that have a proportion >0.8 are then processed using the 'Statistical Analysis (one factor)' feature on MetaboAnalyst 5.0 with the stages of (1) data upload, (2) data integrity checking, (3) data filtering, (4) data normalization ($\log_{10}$) and (5) statistical processing. This statistical process is divided

into three: (5a) Principal Component Analysis (PCA) (score plot and loading plot), (5b) PLS-DA (Partial Least-Squares Discriminant Analysis) (Variable Importance in Projection (VIP) score) and (5c) heatmap.

RESULTS AND DISCUSSION

Growth performance

The chicken used in this study used the Ross 308 strain. This Ross 308 strain performs better than other strains in BW and FCR parameters (Martínez & Valdivié 2021). The total number of samples was 30 for each treatment except BCLP, which had 29 samples due to one bird being affected by the Runting Stunting Syndrome (RSS). RSS in broiler chickens is observed on 4-7 days with shorter shanks, lower body weight, pale, distention of the abdomen, poor feather development, listlessness, and diarrhea (Li et al. 2020; Aviagen 2018).

Assumption tests were conducted to ensure that the data followed a normal distribution pattern and that data variance was homogeneous (Kozak & Piepho 2018; Orcan 2020). The RSD of the avgBW parameter ranges from 7.08 to 11.07 %. Data uniformity is acceptable if the ±RSD value is <10 % (Aviagen 2018). The performance profile of the chickens showed that only avgBW was significantly different after ANOVA and Tukey tests with the highest to lowest weights in order: 0-10 days (LP^{a}, $BCLP^{ab}$, NC^{b}, BC^{b}), 0-21 days: (LP^{a}, $BCLP^{b}$, BC^{b}, NC^{b}), 0-30 days (LP^{a}, BC^{ab}, $BCLP^{ab}$, NC^{b}). The data indicate that treatment with *L. plantarum* yields the best avgBW performance across all rearing periods.

Table 3. Growth performance for parameters avgBW, cumFI, adjFCR, mortalityPEF with RSD values and letter notations from post hoc tests for data significance

Parameters	Treatment	0-10 d	0-21 d	0-30 d
avgBW (g/bird) ±RSD (%) (P≤0.05)	NC	300.57^{b}±10.77	940.67^{b}±10.85	1843.57^{b}±11.57
	LP	319.5^{a}±9.72	1057^{a}±8.72	1999.43^{a}±9.21
	BC	299.5^{b}±8.66	972.33^{b}±7.99	1895.87^{ab}±10.06
	BCLP	299.67^{ab}±10.86	994.69^{b}±9.43	1892.96^{ab}±7.08
cumFI (g/bird) ±RSD (%) (P≤0.05)	NC	313.57±8.49	1232.77±10.31	2553.53±7.21
	LP	330.30±6.14	1336.37±10.57	2748.00±7.77
	BC	316.43±4.93	1257.17±4.01	2605.63±3.73
	BCLP	311.17±2.86	1269.43±6.05	2619.88±4.76
adjFCR ±RSD (%) (P≤0.05)	NC	1.05±7.83	1.33±9.36	1.38±6.14
	LP	1.04±8.18	1.25±8.19	1.33±3.36
	BC	1.06±1.63	1.30±3.86	1.35±2.07
	BCLP	1.05±4.70	1.28±8.66	1.36±7.48
mortality (%)	NC	0	0	0
	LP	0	0	0
	BC	0	0	0
	BCLP	0	0	3.33
PEF ±RSD (%) (p≤0.05)	NC		444.62±9.75	
	LP		485.64±2.75	
	BC		459.88±4.53	
	BCLP		458.57±6.93	

NC= negative control, LP = *Lactobacillus plantarum,* BC= *Bacillus coagulans,* BCLP= *Bacillus coagulans* & *Lactobacillus plantarum,* avgBW= average body weight, cumFI= cumulative feed intake, adjFCR= adjusted feed conversion ratio, PEF= performance efficiency Factor on 0-30 day observation, RSD= relative standard deviation

The non-parametric significance difference test (Kruskal-Wallis) was conducted on several other performance parameters (cumFI, adjFCR, and PEF). These parameters stated that they were not significantly different, as indicated by a P>0.05. However, when viewed from the average data, the LP treatment still has the best value compared to other treatments. One bird in the BCLP treatment was excluded due to stunting, resulting in a mortality rate of 3.33%.

FCR measures feed utilization efficiency or production efficiency; the smaller the FCR value, the better or more efficient (Prakash et al., 2020). Meanwhile, PEF is used to measure overall growth performance, which indicates that the higher the PEF value, the better the growth performance (Aviagen 2018). Ross 308 broilers at 28 and 35 days will have FCR (1.5 and 1.56) and PEF (371.31 and 405.65), respectively (Petričević et al. 2024). The results of this study showed that all treatments had FCR and PEF values better than the performance in large-scale rearing. Comparisons can also be made with the parameter values of the Ross 308 growth standard (Aviagen 2022). Compared to this standard, avgBW values were higher in all treatments for 0-30 days. While cumFI was higher than the standard in all treatments and all rearing days. However, adjFCR had worse results for all treatments and rearing days. Other studies have found that *L. plantarum* can significantly improve chicken growth performance (Banu et al. 2019; Peng et al. 2016; Humam et al. 2019; Wang et al. 2023). Separately, *B. coagulans* has also been shown to improve chicken performance (Zhang et al. 2021; Zhen et al. 2018). A comparison between *L. plantarum* and another endospore-forming probiotic (*B. subtilis*) in broilers (22-98 days old) studied by Nam et al. (2022) showed that treatment with *L. plantarum* resulted in better BW compared to *B. subtilis* while the FCR parameters demonstrate no significant differences, with the best value is observed with *L. plantarum* treatment. At the same time, the study on the effects of *L. plantarum* and *B. coagulans* on broilers (1-42 days) shows that the treatment with *L. plantarum* yields the most favorable outcomes for the Average Daily Gain (ADG) and Average Daily Feed Intake (ADFI) but not FCR parameters (Yu et al. 2022).

Metabolite profile

Principal Component Analysis (PCA) is a method to reduce the dimensionality of specific datasets (Debik et al., 2022). It improves interpretability without losing much information (Hasan & Abdulazeez, 2021). The PCA score plot between the overlapping treatment groups in Figure 1 shows no significant difference between the treatments. If there is no clear separation between groups on the PCA graph, then there is no significant effect between treatments, and it can be considered indistinguishable (Fiehn 2016; Jiang et al. 2022). However, the LP and BC treatment groups visually provide a more oval cluster than the other treatments. PCA loading plot graph serves to visualize the loading contribution of each metabolite to the variance observed in the data between treatments (Withers et al. 2020). The further away from the center, the more influential the metabolite is to the treatment (Ren et al. 2015).

In summary, the loading plot illustrates the direction of projection of the metabolite features of the PCA score plot in space where it has the most extended vector for the highest variation in the data (Van Dyk, 2022). Metabolites that contribute strongly, as seen in the loading plot in Figure 1, are palmitic acid, 1-octadecanol, and 5-oxoproline. Metabolites that indicated a negative correlation were 1-octadecanol and palmitic acid. One comparative study examining the metabolites of the probiotics *B. coagulans* and *L. plantarum* through untargeted metabolomic analysis is reported by Cukkemane et al. (2020). This study utilized various probiotics to ferment different milk media, including four lactic acid bacteria (LAB) and one spore-forming bacterium (SFB), specifically *B. coagulans* ATCC 12425 and *L. plantarum* NRC 716. The PCA and heatmap analysis results of each class of detected compounds indicated that *B. coagulans* and *L. plantarum* metabolites differed significantly. However, while utilizing the same bacteria, this study does not detail the chicken host's metabolite conditions, as it employs milk for fermentation. Zhang et al. (2023) reported significant differences in the PCA analysis of cecum samples from chickens undergoing LAB probiotic treatment in response to heat stress.

The PLS-DA score plot in Figure 2 does not demonstrate a clear separation between treatments. However, the clustering observed indicates that the BC, LP, and NC treatments exhibit distinct patterns and directions. Worley and Powers (2016) state that PLS-DA aggressively enforces separations between experimental groups and is often employed as an alternative method when PCA fails to reveal group separation. However, this practice carries significant risks. Without proper validation, PLS-DA can quickly produce statistically unreliable group separations. Q^2 is the estimated value of a model's predictive ability, calculated through cross-validation. A strong prediction will yield a high Q^2 value; conversely, if Q^2 is negative, the model is deemed non-predictive (Szymańska et al., 2012). This study's PLS-DA model demonstrates positive Q^2 values for three principal components (PCs), precisely 0.10, 0.13, and 0.14. Therefore, it can be concluded that the model has good predictive capability.

VIP (Variable Importance in Projection) is a parameter used to calculate a cumulative measure of the

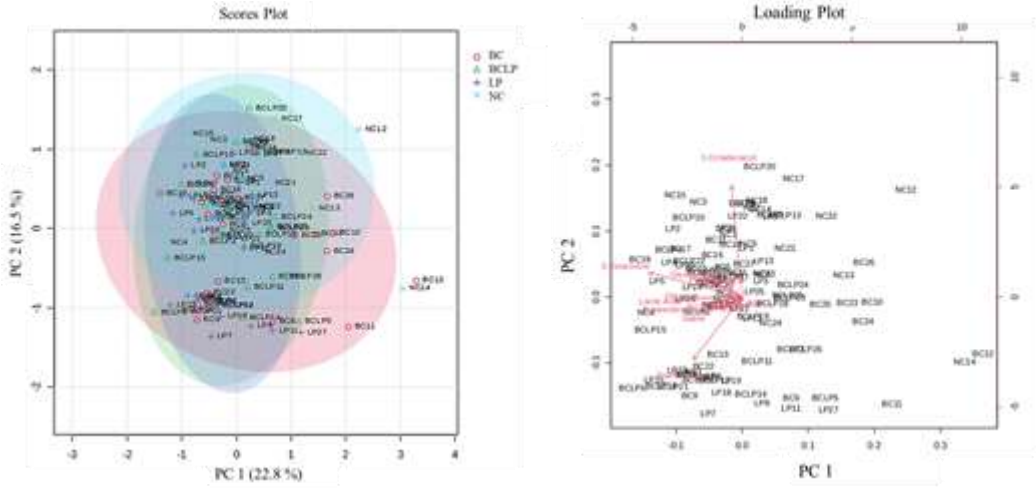

Figure 1. The PCA score plot (left) and loading plot (right) illustrate the differences between treatments and influential metabolite variance

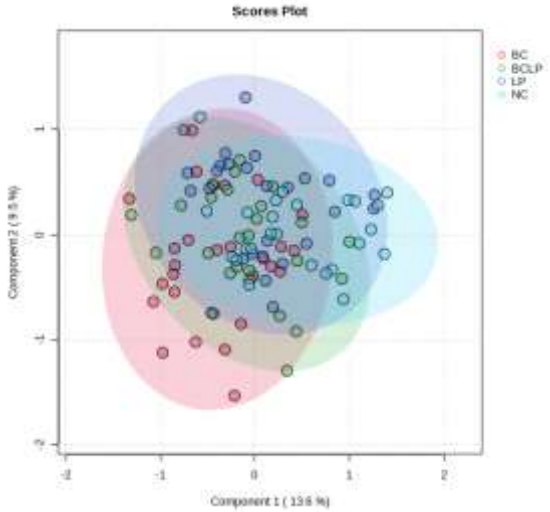

Figure 2. The PLS-DA score plot illustrates the enforced separation between treatments. Clustering indicates that the BC, LP, and NC treatments exhibit distinct patterns and directions but do not demonstrate clear separation

influence of individual variables on the model (Galindo-Prieto et al., 2014). This analysis reflects the loading weight for each component and the response variability explained by the PLS-DA components that can be used for feature selection (Thevenot 2016; Zheng et al. 2023). Metabolites (features) with VIP values >1 in PLS-DA models are identified as important differential metabolites (Deng et al. 2021; Gromski et al. 2015). Mapping metabolites between treatments on a heatmap provides an overview of the hierarchical clustering of metabolite profiles (Vacanti 2019). Heat maps allow users to easily visualize changes in metabolite concentration patterns across samples and treatments, displaying actual data values using color gradients (Chong & Xia 2020). The heatmap dendrogram in Figure 3 shows that LP treatments are grouped with BC and continue to be further grouped with NC. BC provided the most distinct profile compared to the other treatments. Separation of important metabolites is done using VIP analysis in PLS-DA. Metabolite screening based on VIP score >1 in Figure 3 resulted in palmitic acid, 1-octadecanol, acetic acid, lactic acid, and butanoic acid as important metabolites. Broiler chickens with poor performance are indicated by the increase of several metabolites in the cecum, namely D-mannose, hexadecanoic acid, cholesterol, L-valine, L-leucine, glutamic acid, glucopyranose, α-D-allopyranose and phosphoric acid (Chen et al. 2021). In this study, it was described as increasing 1-octadecanol and glycolic acid.

Relationship between chicken performance and metabolite profile

Rinttilä & Apajalahti (2013) summarize that metabolites derived from microbiota composition can influence growth performance and suggest that the

cecal microbial profile may reflect the efficiency of feed digestion and nutrient absorption in the intestine. The relationship between chicken performance and metabolite profiles can be summarized in Table 4. LP and BC profiles have significant metabolite differences and significant avgBW performance differences, particularly for 0-21 rearing days. The primary metabolites differentiating between LP and BC quite far from the white mid-spectrum were acetic acid, lactic acid, and butanoic acid. The BC treatment also had a significantly lower concentration than NC. Differentiating metabolites that are indicators of unsupplemented by probiotics are the decrease of palmitic acid and the increase of 1-octadecanol. Metabolites expressed as differentiating metabolites (biomarker candidates) are lactic acid as a good avgBW performance distinguisher and 1-octadecanol and palmitic acid as distinguishers without probiotic supplementation. Xing et al. (2020) reported changes in unique compounds that could serve as biomarkers in the digestive tract of laying hens supplemented with *B. coagulans* and exposed to lead (Pb). These changes included the presence of antioxidant and antibacterial compounds, such as 4-acetamido butanoic acid, dodecanoic acid, L-3-phenylacetic acid, apigenin, and daidzein. Zhang et al. (2021) found an increase in SCFA compounds such as acetic acid, propionic acid, butyrate, isobutyric acid, and valeric acid in the digestive tract of broiler chickens administered *B.* coagulans. Additionally, Ito et al. (2022) noted that while the concentrations of certain SCFAs, such as propionate and butyrate, would increase, other types, including acetate and lactate, would decrease.

Analysis of differences in growth performance characteristics in the administration of probiotics *L. plantarum* and *B. coagulans* resulted in only avgBW parameters significantly different with the highest to lowest weights in order LP, BCLP, BC, NC.

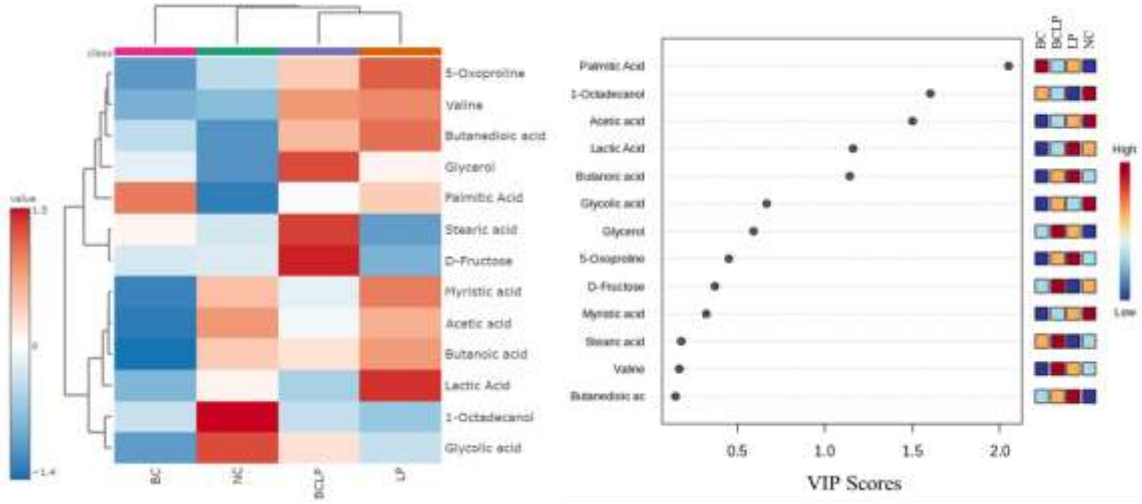

Figure 3. The heatmap (left) and VIP score (right) of identified metabolites with a proportion >0.8 illustrate the hierarchical grouping of treatments and metabolites based on their relative levels.

Table 4. Relationship mapping between significant performance parameters (avgBW) and important metabolites with a VIP score >1

				avgBW				
0-10 day	LP^{a}	>	BCLP^{ab}	>	BC^{b}	>	NC^{b}	
0-21 day	LP^{a}	>	BCLP^{b}	>	BC^{b}	>	NC^{b}	Metabolite
0-30 day	LP^{a}	>	BCLP^{ab}	>	BC^{ab}	>	NC^{b}	
		a		*b*		*a*		*b*	Palmitic acid
		b		*b*		*b*		*a*	1-Octadecanol
		a		*a*		*b*		*a*	Acetic acid
		a		*b*		*ab*		*ab*	Lactic acid
		-		-		-		-	Butanoic acid

NC= negative control, LP= *Lactobacillus plantarum*, BC= *Bacillus coagulans*, BCLP= *Bacillus coagulans* & *Lactobacillus plantarum*. Diferent superscript letters mean significant different. Treatments that do not have the same letter notation are significantly different (α= 0.05).

Other performance parameters (adjFCR, cumFI, and PEF) did not differ significantly. Metabolite profile analysis on the administration of probiotics *L. plantarum* and *B. coagulans* in the digestive tract of broiler chickens with NC, LP, BC, and BCLP treatments gave results that were not significantly different after PCA analysis. Important metabolites with VIP score >1 are acetic acid, lactic acid, butanoic acid, 1-octadecanol and palmitic acid. Metabolites expressed as distinguishing metabolites between LP and BC are acetic acid, lactic acid, and butanoic acid. At the same time, metabolites expressed as distinguishing metabolites of biomarker candidates are lactic acid as a good avrBW performance distinguisher and 1-octadecanoic and palmitic acid as a distinguisher without probiotic supplementation.

CONCLUSION

The administration of probiotics *Lactobacillus plantarum* (LP) and *Bacillus coagulans* (BC) significantly influenced broiler growth performance, with average body weight (avgBW) being the only parameter showing significant differences. The highest to lowest avgBW values were observed in the order of LP, BCLP, BC, and NC treatments. Other performance parameters, including adjFCR, cumFI, and PEF, showed no significant differences. Metabolite profile analysis indicated no significant differences between treatments based on PCA and PLS-DA results. However, important metabolites with a VIP score >1 were identified, including acetic acid, lactic acid, butanoic acid, 1-octadecanol, and palmitic acid. Acetic acid, lactic acid, and butanoic acid were key distinguishing metabolites between LP and BC. Additionally, lactic acid was identified as a potential biomarker for good avgBW performance, while 1-octadecanol and palmitic acid were differentiating metabolites in treatments without probiotic supplementation. These findings suggest that probiotic supplementation can selectively influence broiler growth performance and metabolite profiles, providing valuable insights for optimizing broiler nutrition strategies.

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