JAST (Journal of Animal Science and Technology) TITLE PAGE

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ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Effect of probiotics on growth performance, cytokine levels, and gut microbiome composition of broiler chickens for 7 and 35 days
Running Title (within 10 words)	Efficacy of probiotics on broilers for 7 and 35 days
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Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This research was funded by the Cooperative Research Program for Agriculture, Science, and Technology Development (Project No. PJ015693), Rural Development Administration, Republic of Korea, and the 2024 RDA Fellowship Program of the National Institute of Animal Sciences, Rural Development Administration, Republic of Korea.

Acknowledgements	Not applicable.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Jeong JY. Data curation: Kim M. Formal analysis: Kim J. Methodology: Park C. Software: Jeong JY, Kim M. Validation: Song J, Kim DW, Park NG. Investigation: Kim YB. Writing - original draft: Jeong JY, Kim J. Writing - review & editing: Jeong JY, Kim J, Kim M, Kim YB, Park C, Song J, Kim DW, Kim M, Park NG.
Ethics approval and consent to participate	All animal procedures were reviewed and approved by the National Institute of Animal Science (NIAS) Animal Use and Care Committee in Korea (NIAS-2021-0508).

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Abstract

Through microbial fermentation, probiotics are essential for improving growth performance and gut health in broiler chickens. This study aimed to assess the effects of three additives on growth performance, cytokine levels, and cecal microbiota in broiler chickens. One-day-old Arbor Acres chicks (total 300) were randomized into four groups: (1) control: basal diet, (2) BS: *Bacillus subtilis* + basal diet, (3) EO: essential oil + basal diet, and (4) BV: *Bacillus velezensis* + basal diet. All chickens were fed and watered ad libitum throughout the experiment. Feed intake and body weight were measured weekly. On days 7 and 35, cecal contents of one bird per replicate, based on average body weight, were collected and analyzed for microbiota using 16S rRNA gene amplicon sequencing. The BS group exhibited enhanced growth performance, including increased final body weight, average daily gain, and reduced feed conversion ratio compared to that of the other groups. On day 7, the BS group exhibited a higher abundance of *Eisenbergiella* (8.24 %), and on day 35, there was an increased abundance of *Firmicutes* (99.63 %) and *Lachnoclostridium* (1.4 %). These results indicate that *B. subtilis* may be a promising probiotic for enhancing broiler health by modulating gut microbiota.

Keywords: probiotics; broiler chickens; growth performance; gut microbiota; *Bacillus subtilis*

Introduction

The gut is essential for nutrient absorption, and the development of the intestinal system can enhance nutrient absorption, growth performance, and animal health [1]. The poultry's digestive tract contains many microorganisms, commonly called microbiota. The gut microbiome regulates gut health. A balanced gut microbial population enhances feed digestibility and efficiency, thereby enhancing growth and feed conversion [2]. Various feed additives in poultry diets can affect the gut microbiome, with a few specifically used to modulate the gut microbiome [3].

For decades, antibiotics have been used in the poultry industry to improve production, growth, and health, thereby increasing economic benefits [4, 5]. Although antibiotics are crucial for combating bacterial infections, they can also have unintended consequences. For example, they can result in increased antibiotic resistance, food and egg contamination, and environmental pollution. Therefore, in 2006, the European Union banned non-therapeutic antibiotics for growth and production. Korea adopted a similar approach in 2011 [6]. Consequently, there is a need to develop alternatives to antibiotics, such as probiotics, to ensure the continued efficacy of antimicrobial agents.

Probiotics are defined as living microorganisms that, when administrated in adequate amounts, confer a health benefit on the host and are widely used as feed additives in the poultry industry to improve health and welfare [7]. They offer numerous benefits, including stimulation of host intestinal microorganisms and immune modulation [8]. Recently, probiotics have gained popularity in the poultry industry as substitutes for antibiotics in nutritional supplements and feed additives [9]. These universal feed additives can be combined with other additives to improve the performance and health of poultry [10]. Their beneficial effects are observed directly in the gastrointestinal tract and indirectly through poultry immunomodulation [11]. Probiotic-fed flocks exhibit enhanced laying performance and egg quality, increased daily gain, and improved FCR [12,13]. Additionally, probiotics accelerate the maturation of gut microbiota in broiler chickens [14,15]. Therefore, probiotics have the potential to enhance the productivity and overall health of the poultry industry.

Bifidobacteria, lactobacilli, and *Saccharomyces boulardii* are the most commonly used microorganisms in the production of probiotics. Probiotics are more effective in controlling microorganisms and less harmful to the environment compared to antibiotics. In poultry, probiotics can enhance growth performance and health by improving feed intake and efficiency, maintaining gut integrity, and promoting gut health [11]. Among these, *Bacillus*-based probiotics are particularly effective in enhancing health [16]. *Bacillus* spp. are used to improve production efficiency, boost the immune system, alter the intestinal environment, address metabolic and inflammatory problems, improve cholesterol profiles, and prevent or treat autoimmune diseases in broiler chickens

[17]. These strains support chickens' growth, digestion, and overall health, promoting a healthy gut [18]. Among them, *B. subtilis* has attracted significant attention in probiotic supplementation research due to its high resistance and survival in the hostile environment of the gastrointestinal tract [19]. Additionally, *B. velezensis* is known to facilitate plant growth by inhibiting plant pathogens, and its potential as a probiotic in animal feed has also been evaluated [20].

Essential oils (EOs) are volatile, aromatic compounds synthesized by plants with antimicrobial, antifungal, anti-inflammatory, antioxidant, and antiviral properties [21,22]. Due to these characteristics, several studies have demonstrated their potential as alternatives to antibiotics [23,24]. In particular, essential oils have been shown to improve gut health, thereby enhancing growth performance and immune function in broilers [25,26] Additionally, the supplementation of EOs has been shown to enhance immune capacity and positively affect carcass characteristics in broilers. In this context, future studies should focus on identifying novel strategies to maintain animal health and well-being. Therefore, this study aimed to assess the effects of probiotic and EO supplementation on the growth performance and microbiome composition of broiler chickens.

Materials and Methods

Animal design and sampling

All animal procedures were reviewed and approved by the National Institute of Animal Science (NIAS) Animal Use and Care Committee in Korea (NIAS-2021-0508). A total of 300 one-day-old broiler chicks (Arbor Acres, AA) were purchased from a commercial farm, sorted by sex (male), and weighed. Subsequently, the chicks were randomized into four experimental groups (1) control: basal diet, (2) BS: *Bacillus subtilis* (3 mg/kg) + basal diet, (3) EO: essential oil (3 mg/kg) + basal diet, and (4) BV: *Bacillus velezensis* (3 mg/kg) + basal diet. The oregostim used in the EO test is a natural oregano oil extract with antibacterial, antioxidant, and gut-regenerative properties. It was purchased from SOLTON (Seoul, Korea). Each group was housed in 12 replicate cages (1.5 m x 0.9 m x 0.5 m) containing 6 – 7 birds and were reared for 35 days. The chicks received starter, grower, and finisher diets at 1, 2 – 3, and 4 – 5 weeks, respectively. Throughout the experiment, all chickens were fed and watered *ad libitum.* The light period was 24 hours at 40 lux from 0 to 7 d, then 19 hours at 20 lux from 8 to 35 d. The experimental environment was controlled at 33 ± 1 ℃ and 50% relative humidity, then reduced by 2 ℃ each week to 24 ℃. Room temperature was measured daily to ensure consistency. The temperature was manually monitored and controlled. Thermometers and manual ventilation were used. This method is labor-intensive but effective. The ventilation

system was activated to remove contaminated air and introduce fresh air. A low-power heater and humidifier were installed. The lighting system was put on a timer. Windows were insulated, and curtains were installed. On days 7 (n = 10) and 35 (n = 12), cecal contents were collected from one bird per replicate selected based on the average body weight for microbiome analysis. The control diet of the mesh type was based on maize and soybean meal (Table 1). Feed consumption and body weight per cage were measured weekly and weight gain and FCR were calculated for mortality. At 7 and 35 days of age, the chickens in the treatment groups were euthanized under carbon dioxide anesthesia. Blood was collected from the wing vein at 35 days. Cecal digesta were frozen in liquid nitrogen and stored at -80 °C.

Hematology and cytokine analysis

Blood samples were collected from wing vein into ethylenediaminetetraacetic acid (EDTA tubes; BD Vacutainer). Hematological parameters were assessed using a Mindray BC-5300 automated hematology analyzer (Mindray Co., Ltd., Shenzhen, China). The concentrations of pro-inflammatory cytokines, including interleukin 1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α), were measured using commercially available chicken enzyme-linked immunosorbent assay kits (EK780087, EK780053, and EK780062; AFG Scientific), following the manufacturer's protocol.

DNA preparation and microbial community analysis

DNA from cecal samples was extracted using the bead-beating plus column method with a QIAamp DNA kit (Qiagen, Hilden, Germany). The samples were prepared for PacBio instrument sequencing following the single-molecule real-time (SMRT) bell template preparation guide. SMRTbell libraries were constructed by ligating hairpin adapters to double-stranded DNA ends, followed by annealing sequencing primers and polymerase to the library for SMRT sequencing using Sequel II Binding Kit 2.1 and Sequel II DNA Internal Control Complex 1.0 (PacBio, USA). For bacterial 16S rRNA sequencing, primers 27F (5′- AGRGTTYGATYMTGGCTCAG -3') and 1492R (5′- RGYTACCTTGTTACGACTT -3') were used to amplify the full-length variable regions of the gene, resulting in a single amplicon of approximately 1,400 base pairs (bp). Polymerase chain reaction (PCR) amplification involved 25 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. After cleanup, eight additional cycles were performed to attach the adapters. Negative controls were included.

The resulting amplicons were sequenced by Macrogen (Seoul, Korea) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) as previously described. We assessed the size of PCR-enriched fragments using an Agilent Technologies 2100 Bioanalyzer with a DNA 1000 chip. To ensure accurate data, we optimized cluster density in our prepared libraries using qPCR, following the Illumina guidelines. DADA2 (v1.20.0) was used to process Illumina sequences. First, primer sequences were removed and reads were trimmed based on length. Those with >5 expected errors were removed. The remaining reads were dereplicated and analyzed for sequencing errors using loessErrfun. True sequence variants were inferred, and forward and reverse reads were merged to obtain complete denoised sequences. Chimeric amplicon sequence variants (ASVs) were identified and removed. The remaining reads were annotated using the Silva v.138.1 database.

Pacific Bioscience data were demultiplexed, and consensus circular sequences (CCS) were generated using the SMRT-Link analysis software (version 9). A mean of 19 high-fidelity passes were used. Subsequently, the obtained CCS underwent quality checks using the DADA2 R Statistics package (v1.20.0). Chimeric ASVs were removed, and the remaining reads were annotated using the naive Bayesian classifier from DADA2 against the Silva138.1 database that comprises the species training set from the Silva138 database. An optimal match is designated only when the discrepancy between the initial and secondary optimal matches exceeds 2 %. The taxonomic annotations were subsequently used to generate contingency tables for each taxonomic rank.

Statistical analysis

Linear discriminant analysis (LDA) effect size (LEfSe) was used to analyze taxon profiles for differential abundance among the four treatment groups (LDA score > 3). Permutational multivariate analysis of variance (PERMANOVA) was used to compare beta diversity analysis and functional genetic profiles among the four treatment groups. Rarefaction curves, richness, and diversity analyses were performed using the minimum number of reads annotated at the ASV. Principal Coordinate Analysis (PCoA) was performed on Bray-Curtis distances to assess similarities between sample types and platforms. Analysis of variance was performed using PERMANOVA on distance matrices from the Vegan R package. Growth performance and blood analysis used two-way and one-way analyses of variance, respectively, with a post-hoc Tukey's test. Significant differences ($p < 0.05$) were determined using Prism software (Ver 9.5.1).

Results

Growth performance in broiler chicken fed three different additives

Figure 1 illustrates probiotic treatment's effects on broiler chickens' growth performance. Initial body weights (40.0 ± 0.05 g) were similar among all dietary treatment groups. BW and ADG showed no significant difference among all groups for 7 days. However, the FCR exhibited an increase ($p < 0.05$) in all treatment groups compared to the control during the starter period (0 to 7 d). In the growing phase (8 to 21 d), the BW, ADG, and FCR were not significantly different in all diet groups. During the finishing phase (22 to 35 d), the final body weight of the BS group (1,947 ± 29.9 g) was significantly higher than that of the other groups, including the control (1,807 ± 24.4 g), EO (1,860 ± 46.6 g), and BV (1,821 ± 27.6 g) groups ($p < 0.05$). The BS group also had the highest ADG at the end of the study compared to the other groups ($p < 0.05$). The BS group had the lowest FCR compared to the other groups ($p < 0.05$).

Serum biochemical analysis and cytokine levels of broiler chicken fed three different additives

Figure 2 illustrates the effects of probiotic treatment on the hematological and cytokine parameters of broiler chickens at 35 days. Cytokine parameters (TNF-α, IL-1B, and IL-6) were not significantly different among the probiotic-treated groups (Figure 2A). Additionally, hematological parameters, including WBC, RBC, hemoglobin, mean corpuscular volume, and platelet counts, exhibited no significant differences among the probiotic-treated groups (Figure 2B).

Cecal microbiota composition of broiler chicken supplemented three different additives

Alpha diversity of the cecal microbiota was assessed using the observed and the Chao 1 indices to analyze the effects of age and the four dietary treatments. Additionally, beta diversity was analyzed using PCoA with the Bray–Curtis index to assess differences in the microbial community composition. No significant differences in alpha or beta diversity were observed between the three probiotic treatments. However, the observed and Chao 1 indices were significantly higher in 35-day-old broiler chickens than that in the 7-day-old broiler chickens ($p < 0.001$; Figure 3A and B). PCoA-based beta diversity analysis using the Bray–Curtis index indicated a distinct separation between the microbial communities of 7-and 35-day-old broiler chickens ($p < 0.001$; Figure 3C).

Cecal microbiota composition of broiler chickens supplemented with B. subtills

Figure 7 illustrates the alpha diversity analysis using the observed and Chao1 indices and beta diversity analysis using PCoA with the Bray–Curtis index in the BS group. The observed and Chao1 indices were significantly higher

in 35-day-old broiler chickens than that in 7-day-old broiler chickens ($p < 0.001$; Figure 7A and B). The Bray–Curtis index indicated a distinct separation between the microbial communities of 7- and 35-day-old broiler chickens ($p < 0.001$; Figure 7C).

Figure 8 illustrates the taxonomic bar plots for the BS group in 7- and 35-day-old broiler chickens, demonstrating their mean relative abundance. At the phylum level, *Firmicutes* were dominant in both 7- (97.75 %) and 35-day-old (99.63 %) broiler chickens (Figure 8A). At the genus level, *Eisenbergiella* (8.24 %), *Ruminococcus torques* group (X), and *Butyricicoccus* (6.38 %) were dominant on day 7, and *Faecalibacterium* (13.98 %), *Clostridia_UCG_014* (X), and *Lactobacillus* (7.84 %) were dominant on day 35. In the BS group, LEfSe analysis demonstrated that 7-day-old broiler chickens exhibited a relative abundance of *Proteobacteria* at the phylum level, whereas 35-day-old broiler chickens exhibited a relative abundance of *Firmicutes* and *Cyanobacteria* (Figure 9A). At the genus level, 7-day-old broiler chickens were relatively abundant in *Eisenbergiella*, *Butyricicoccus*, *Escherichia–Shigella*, *Enterococcus*, *Erysipelatoclostridium*, *Oscillibacter*, *Lachnoclostridium*, *Anaerotruncus*, *Anaeroplasma*, and *Tyzzerella*, whereas 35-day-old broiler chickens were relatively abundant in *Faecalibacterium*, *Lactobacillus*, *Romboutsia*, *Blautia*, *Fusicatenibacter*, *Ruminococcus*, *Gastranaerophilales*, and *Anaerostipes* (Figure 9B).

Discussion

In this study, no significant differences in growth performance were observed among dietary treatments on day 7 post-hatching, except for FCR. Consistent with our findings, several studies have also failed to demonstrate significant effects of supplementing *B. subtilis*, essential oils, and *B. velezensis* on growth performance at day 7 post-hatching [27-29]. Nevertheless, *B. subtilis* supplementation significantly enhanced weight gain and feed efficiency at the finisher phase, contributing to the improved growth performance in broilers. Molnár et al. [30] observed that providing 7.27×10^9 colony-forming units (CFU)/g of B. subtilis-supplemented diets increased body weight and feed conversion ratio (FCR) in broiler chickens from days 7 to 42 post-hatching. Additionally, Amerah et al. [31] demonstrated that supplementing 1.5×10^8 CFU/kg of *B. subtilis* improved FCR in broiler chickens at 42 days post-hatch. Consistent with previous studies, our findings highlight the positive effects of B. subtilis on growth performance. This enhancement when using B. subtilis as a probiotic supplement may result from its role in maintaining a beneficial balance in the intestinal microbiome by increasing the population of beneficial bacteria and inhibiting the growth of pathogenic bacteria [32,33].

However, in this study, supplementation with essential oils and *B. velezensis* did not significantly affect the growth performance of broiler chickens throughout the experimental period. Similarly, Jang et al. [34] supplemented 25 and 50 mg/kg of essential oils in the diet but observed no significant difference in growth performance compared to the basal diet. In contrast, Khattak et al. [35] supplemented 100, 200, 300, 400, and 500 mg/kg of essential oils and found that all treatments improved growth performance compared to the control group after 10 days of age. In our study, 3 mg/kg of essential oil was added to the diet, which may explain the discrepancy in results due to the relatively low concentration. Tsai et al. [36] supplemented broiler chickens with 1.5×10^9 CFU/mL of *B. velezensis* for 35 days but did not observe a significant improvement in growth performance.

In contrast, Zhu La et al. [29] reported that broilers supplemented with 1×10^9 CFU/mL of *B. velezensis* for 42 days exhibited increased daily weight gain after 10 days of age and improved final body weight on day 42. Our study's lack of significant improvement in final body weight may be attributed to the shorter testing period, 7 days less than that of Zhu La et al. [29]. Several studies have also demonstrated that probiotics improve growth performance from 21 days of age and significantly improve body weight at 42 days [27]. Furthermore, this study was conducted under laboratory-scale conditions with a fully controlled environment and feeding regimen, which may have masked the growth-promoting effects of the additives. Jang et al. [34] suggested that dietary antibiotic replacements may fail to induce improvements in growth-related parameters under well-nourished and highly controlled conditions. Thus, the effects of probiotics on growth performance, feed conversion, and productivity in farm animals remain inconsistent under certain conditions, potentially rendering their use economically unviable in specific situations [37]. Consequently, further studies focusing on appropriate dosage and experimental duration are required to validate the efficacy of essential oils and *B. velezensis* on growth performance compared to previous studies.

In general, adequate probiotic supplementation can enhance the intestinal mucosa, providing a major barrier against pathogens [38]. These effects may contribute to various aspects of the immune response, such as regulating cytokine production [39,40]. In this study, we assessed the cytokine-modulating ability of *B. subtilis*, essential oils, and *B. velezensis* supplementation in the diet of broiler chickens and observed no significant enhancement. Additionally, blood cell analysis revealed no effects of dietary treatments. Moreover, these additives do not significantly affect the hematology and cytokine regulation in broiler chickens [41]. In contrast to our results, cytokines, including TNF-α, IL-1β, and IL-6 were significantly upregulated by *B. subtilis* supplementation [42]. Moreover, essential oil supplementation increased cytokine levels, such as IL-1β and IFN-γ [43]. Furthermore, *B.*

velezensis regulates various cytokines, such as TNF-α, IL-1β, IL-6, and IL-10 in the blood of broiler chickens [38]. However, the studies above and our study differed in dosage, experimental period, and rearing environment (presence of litter, temperature). These differences may explain the controversial results in blood biochemical parameters [44,45]. Furthermore, in well-controlled experimental settings, reduced corticosterone levels in broilers can enhance humoral immunity by promoting the production of anti-inflammatory cytokines and immunoglobulins [46]. This may have mitigated the additive's effects on immunological parameters in this study. Therefore, further studies are required to clarify the efficacy of these additives on broiler chickens' immune regulation ability.

The intestinal microbiota of broiler chickens develops throughout the gastrointestinal tract and plays a crucial role in maintaining health while also influencing productivity [47]. These beneficial microbes support gut health by aiding feed digestion, nutrient absorption, immune system development, and pathogenic bacterial growth inhibition [48,49]. In this study, no significant differences were observed in alpha or beta diversity in the cecal content between the control and treatment groups. Similar to our findings, previous studies have shown that supplementation with *B. subtilis*, essential oils, and *B. velezensis* did not significantly affect alpha diversity or beta diversity in broiler chickens [33, 50, 51]. The dynamic diversity of the gut microbiome is known to be influenced by diet and age. Still, age has been shown to have a more significant impact than feed additive supplementation [33, 52]. Indeed, the microbial diversity and community composition varied significantly between 7- and 35-day-old broiler chickens across all dietary treatments. Similar patterns were observed in the BS group. Microbial diversity increases with age and forms distinct clusters in the microbiota [48,53]. Our results demonstrated that *Firmicutes* was the most dominant phylum across all treatments, aligning with previous studies demonstrating that *Firmicutes* generally comprise the majority of the cecal microbiota for short-chain fatty acid production in broiler chickens [54,55]. At the genus level, the *Ruminococcus torques* group was dominant in 7-day-old broiler chickens, whereas *Faecalibacterium* was most abundant in 35-day-old broiler chickens. Similarly, at the genus level, the *Ruminococcus torques* group was dominant in 21-day-old broiler chickens, with *Faecalibacterium* becoming more prevalent in 39-day-old broiler chickens [56]. LEfSe analysis demonstrated differentially abundant taxa between the age groups. Host age affects the diversity and stability of microbiota. In broiler chickens, the gut microbiota is dynamic during the first few weeks of life, transitioning to a mature and stable state after 21 days of age [14,57]. In this study, *Proteobacteria* were relatively abundant in the early stages, and the abundance of *Firmicutes* increased with age, aligning with the previous studies [52,57,58]. At the genus level, *Eisenbergiella*, *Butyricicoccus*, and *Escherichia–Shigella* were relatively abundant at 7 days of age. *Eisenbergiella* and *Butyricicoccus* are significant

producers of butyric acid, an energy source for fast-growing broiler chickens [57,59]. Additionally, *Escherichia–Shigella* is a rapidly colonizing microbial group that can dominate the gut of early broiler chickens [60]. However, *Escherichia–Shigella* can cause diseases, such as colibacillosis and shigellosis, highlighting the significance of an early hatching environment in preventing harmful microorganisms [60]. *Faecalibacterium*, which was most abundant at 35 days of age in our study, is known to dominate the mature microbiota after 21 days [55]. Additionally, this study included two feed transitions: from starter to grower feed, and subsequently to finisher feed. These dietary alterations have likely contributed to age-related shifts in the intestinal microbiota [56]. In summary, these findings indicate that the age of broiler chickens significantly affects the composition and diversity of their intestinal microbiota.

Our findings demonstrated that *B. subtilis* supplementation increased the abundance of beneficial microorganisms in broiler chickens compared to other dietary treatments. On day 7, *B. subtilis* supplementation resulted in a relative dominance of *Eisenbergiella*. The abundance of *Eisenbergiella* was lower in the non-*B. subtilis*-treated group compared to that in the *B. subtilis*-treated group. *Eisenbergiella* plays a crucial role in producing butyric acid, which is the preferred energy source for intestinal epithelial cells [59]. Additionally, increasing the abundance of *Eisenbergiella* can improve feed efficiency and reduce FCR in broilers [61]. By day 35, *B. subtilis* supplementation, compared to other dietary treatments, increased the abundance of *Firmicutes* and *Lachnoclostridium* at the phylum and genus levels, respectively. *Firmicutes* are essential for growth that break down indigestible polysaccharides, facilitating nutrient absorption [62,63]. In this regard, the abundance of Firmicutes was shown to improve ADG and reduce FCR in broiler [64]. *Lachnoclostridium* can ferment dietary fiber by breaking down various indigestible polysaccharides and producing butyric and acetic acids [65]. Moreover, a previous study showed that the diet group enriched with *Lachnoclostridium* had improved ADG [64]. *B. subtilis* is known for maintaining the intestinal microbial ecosystem by enhancing mucosal immunity and regulating intestinal commensal microorganisms [66]. This may promote the growth performance broilers and may provide the basis for our study results showing improved body weight and FCR in BS group broilers. Therefore, our results demonstrate that *B. subtilis* supplementation modulates commensal microbiota and supports the findings of previous studies.

Our study assessed the effects of three dietary treatments *B. subtilis*, essential oils, and *B. velezensis* on the growth performance, cytokine levels, and gut microbiome composition of broiler chickens over five-weeks. We observed that *B. subtilis* supplementation enhanced the growth performance of broiler chickens and increased the abundance of beneficial microorganisms throughout their life cycle. This highlights its potential as a promising probiotic to

enhance broiler health. Additionally, we observed age-related alterations in the gut microbiome composition, indicating the significance of growth and health management throughout the broiler life cycle. However, the study did not reveal any significant effects of the three dietary treatments on the immune regulatory ability of broiler chickens, which may be attributed to various complex factors. Therefore, further studies—considering various factors—are required to fully understand the effects of probiotics on the immune capacity of broiler chickens.

Acknowledgments

Not applicable

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Table 1. Feed ingredients for broiler chickens at different growth periods

	Starter	Grower	Finisher
Ingredient			
Maize (%)	53.95	37.95	52.75
Wheat grain (%)	0.00	20.00	15.00
Soybean meal (%)	38.46	33.25	20.12
Corn gluten meal (%)	0.00	0.00	5.45
Soybean oil (%)	3.00	5.00	3.00
Methionine (%)	0.19	0.46	0.39
L-Lysine (%)	0.31	0.23	0.42
L-Threonine (%)	0.00	0.10	0.11
Mono-Dicalcium phosphate (%)	1.90	1.50	1.26
Limestone (%)	1.44	0.76	0.75
Salt (%)	0.25	0.25	0.25
Vitamin premix* (%)	0.50	0.50	0.50
Total (%)	100.00	100.00	100.00
Calculated nutrient value			
Dry matter (%)	87.08	87.42	87.16
Metabolizable energy (kcal/kg)	2886.68	3051.18	3101.88
Crude protein (%)	22.00	21.51	19.50
Crude fat (%)	5.68	7.39	5.74
Crude fiber (%)	3.01	2.86	2.55
Crude ash (%)	6.59	5.30	4.47

* Vitamin premix (kg-1): vitamin A (6250000 IU), vitamin D3 (1000000 IU), vitamin E (15000 IU), vitamin K3 (1000 mg), vitamin B1 (500 mg), vitamin B2 (2500 mg), vitamin B6 (2500 mg), vitamin B12 (10 mg), pantothenic acid (600 mg), nicotinic acid (15000 mg), folic acid (500 mg), biotin (35 mg), choline chloride (150000 mg), iron (20000 mg), copper (2500 mg), zinc (25000 mg), manganese (15000 mg), iodine (600 mg), cobalt (400 mg), and butylated hydroxytoluene (anti-oxidant, 125000 mg).

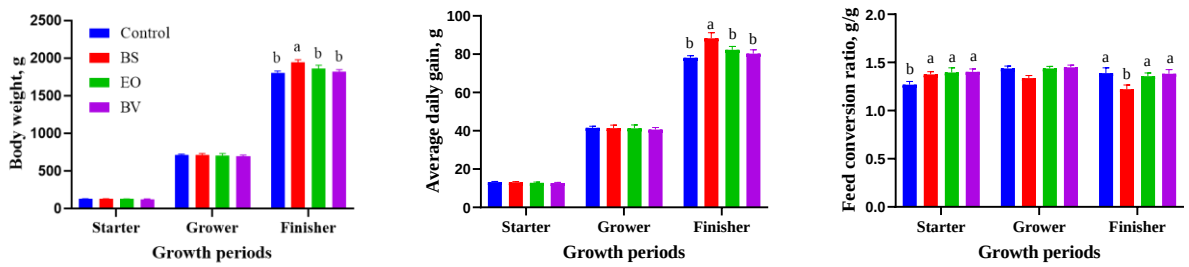
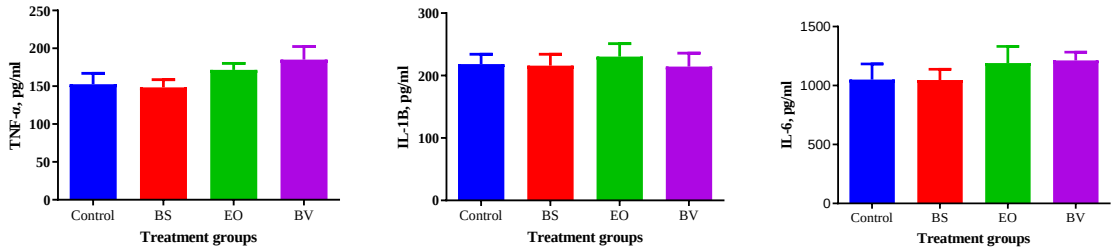


Figure 1. Growth performance of broiler chickens treated with additives, such as *Bacillus subtilis*, essential oils, and *Bacillus velezensis* for growth phases. Data are presented as the mean and standard error of the mean. (A) body weight. (B) Average daily gain. (C) Feed conversion ratio. Control, basal diet; BS, *B. subtilis* + basal diet; EO, essential oil + basal diet; and BV, *B. velezensis* + basal diet. Similar lowercase (e.g., a, b) letters indicate no significant differences, and different letters indicate significant differences ($p < 0.05$) using two-way analysis of variance (ANOVA) with post-hoc Tukey honest significant difference. Three phases: starter (0 to 7 d), grower (8 to 21 d), finisher (22 to 35 d).

A



B

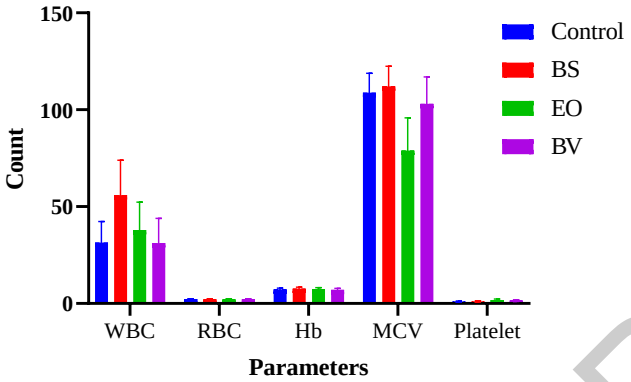


Figure 2. Cytokine levels and hematological analysis of broiler chickens treated with additives, such as *Bacillus subtilis*, essential oils, and *Bacillus velezensis* at 35 days. Data are presented as the mean and standard error of the mean (n = 48). (A) Tumor necrosis factor-alpha (TNF-α). (B) Interleukin-1 beta (IL-1β). (C) IL-6. Control, basal diet; BS, *B. subtilis* + basal diet; EO, essential oil + basal diet; BV, *B. velezensis* + basal diet; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; and MCV, mean corpuscular volume. For statistical analysis, one-way analysis of variance (ANOVA) with post-hoc Tukey honest significant difference used to compare each group.

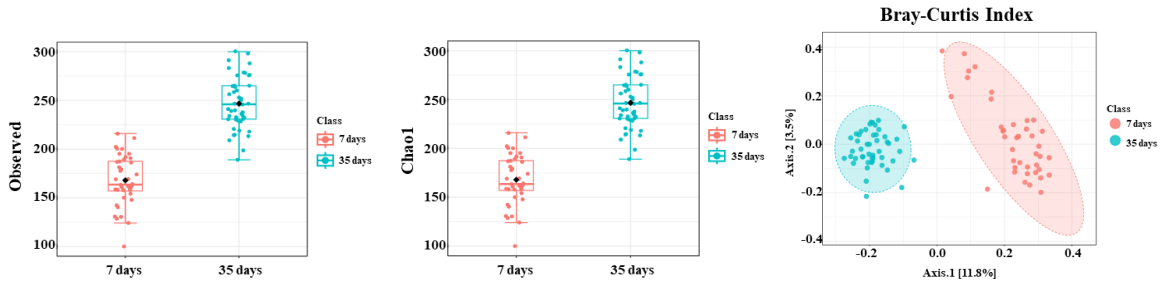
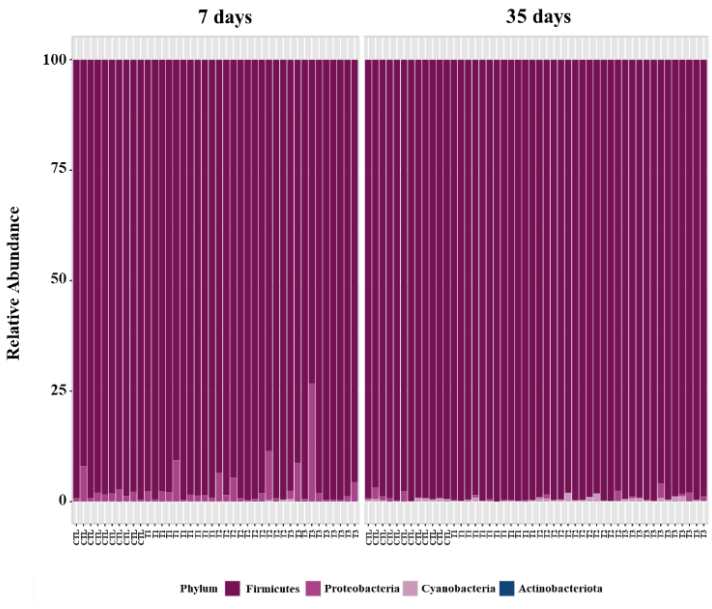


Figure 3. Alpha and beta diversity indices analyzed from the cecal contents of 7- and 35-day-old broiler chickens in all treatment groups. (A) Alpha-diversity index using observed index ($p < 0.001$). (B) Alpha-diversity index using Chao 1 index ($p < 0.001$). (C) Beta-diversity using principal coordinates analysis (PCoA) with the Bray–Curtis index ($p < 0.001$). 7 days, 7-day-old broilers in all groups; 35 days, 35-day-old broilers in all groups.

A



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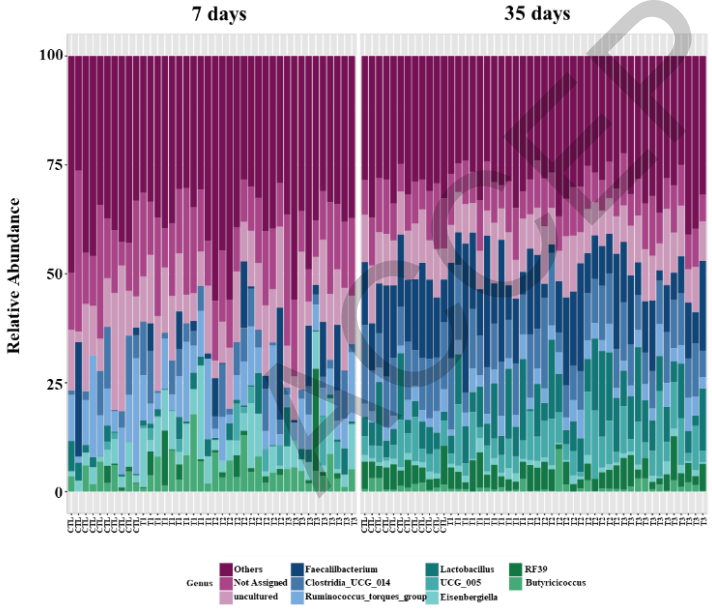
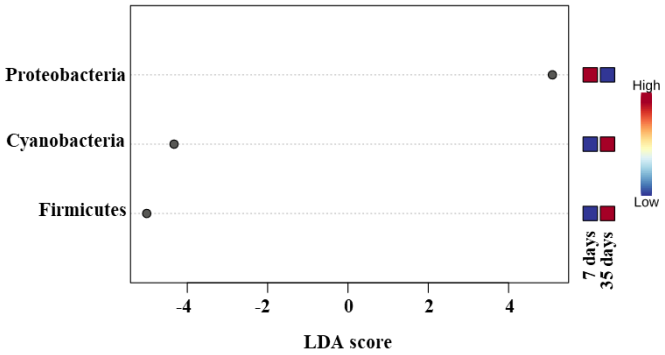


Figure 4. Profiles of cecal microbiota at (A) the phylum and (B) genus levels in broiler chickens supplemented with additives, such as *Bacillus subtilis*, essential oils, and *Bacillus velezensis* on 7 and 35 days. The relative abundance of major phyla in broiler chickens is illustrated. Genera representing < 0.5 % of all sequences across all 48 cecal samples are shown as "Others". Control, basal diet; BS, *B. subtilis* + basal diet; EO, essential oil + basal diet; and BV, *B. velezensis* + basal diet.

A



B

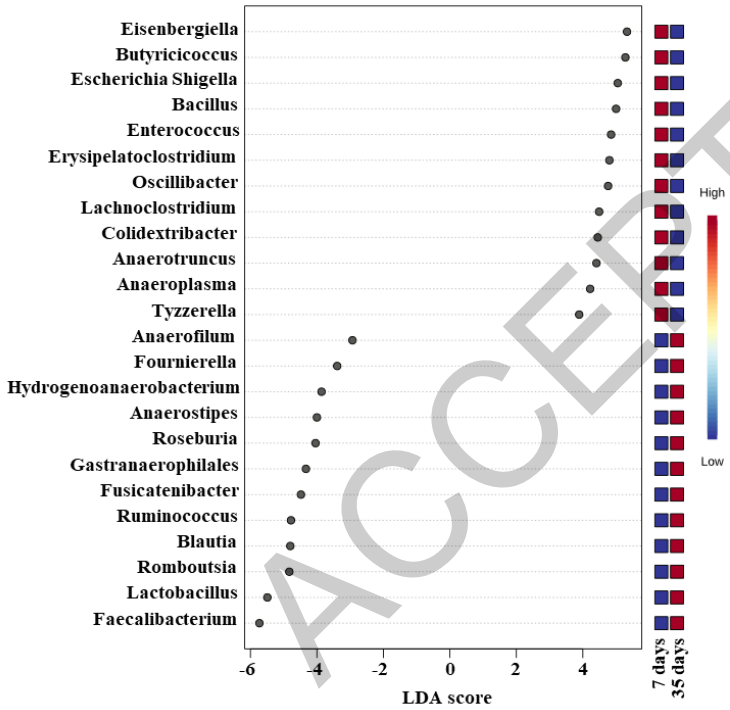
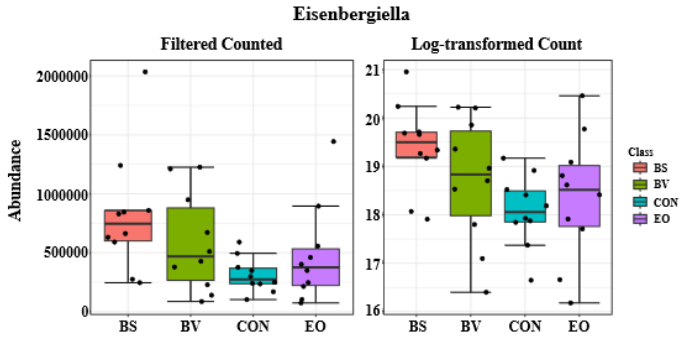
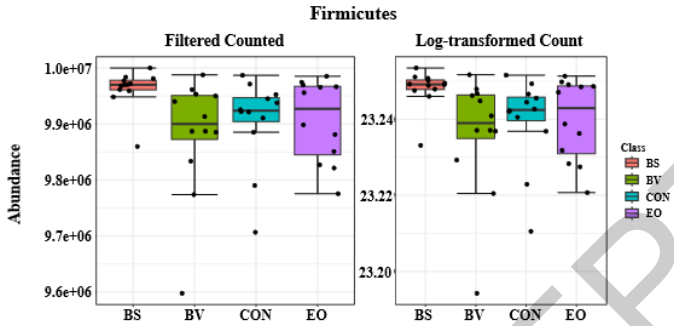


Figure 5. Differentially abundant (A) phyla and (B) genera in 7- and 35-day-old broiler chickens. Linear discriminant analysis (LDA) effect size is used to analyze major phyla and genera that differ in abundance between age groups (LDA score > 4). 7 days, 7-day-old broilers in all groups; 35 days, 35-day-old broilers in all groups.

A



B



C

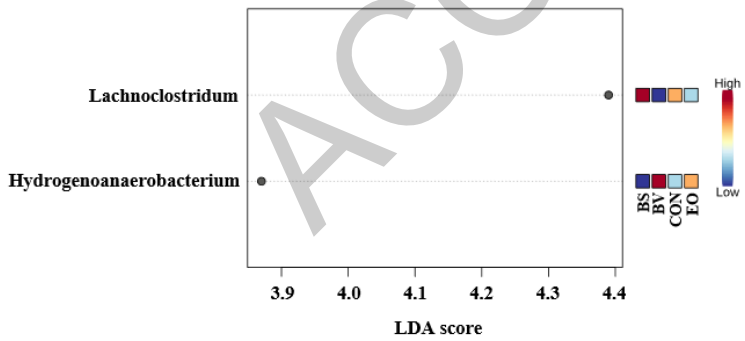


Figure 6. Differentially abundant (A) genera at day 7, (B) phyla at day 35, and (C) genera at day 35 in broiler chickens based on all probiotic treatments. Major phyla and genera that are differentially abundant among the four treatment groups are analyzed using the linear discriminant analysis (LDA) effect size (LEfSe) (LDA score > 4). Control, basal diet; BS, *Bacillus subtilis* + basal diet; EO, essential oil + basal diet; and BV, *Bacillus velezensis* + basal diet.

A B C

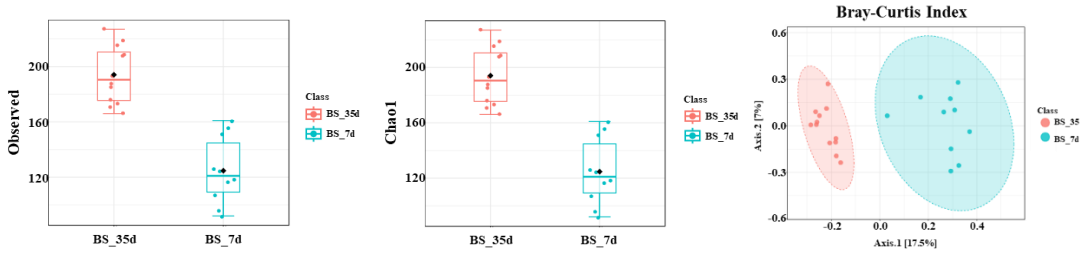
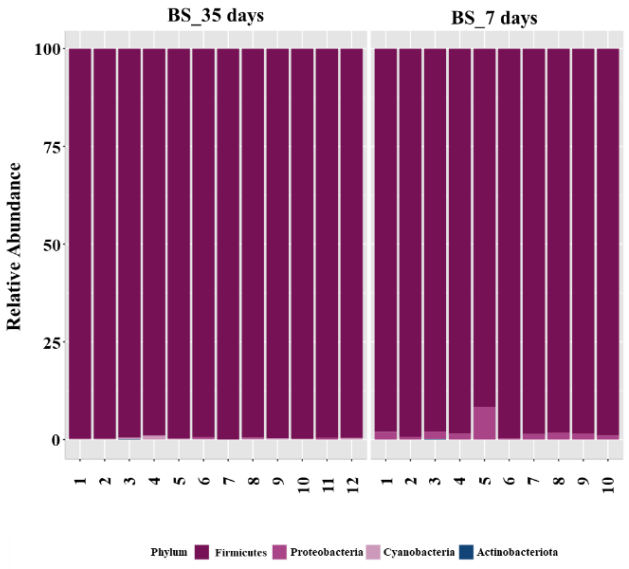


Figure 7. Alpha and beta diversity indices analyzed from the cecal contents of 7- and 35-day-old broiler chickens supplemented with *Bacillus subtilis*. (A) Alpha-diversity index using observed index ($p < 0.001$). (B) Alpha-diversity index using Chao 1 index ($p < 0.001$). (C) Beta-diversity using principal coordinates analysis (PCoA) with the Bray–-Curtis index ($p < 0.001$). BS_.: 7-day-old broiler chickens from the group supplemented with *B. subtilis* and BS_35d: 35-day-old broiler chickens from the group supplemented with *B. subtilis.*

A



B

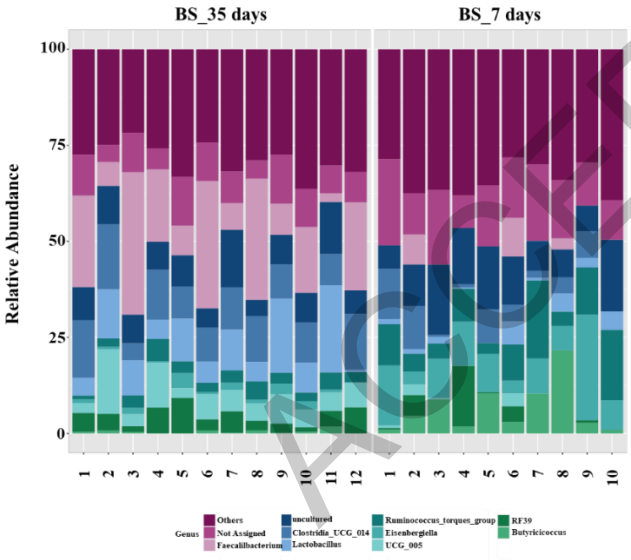
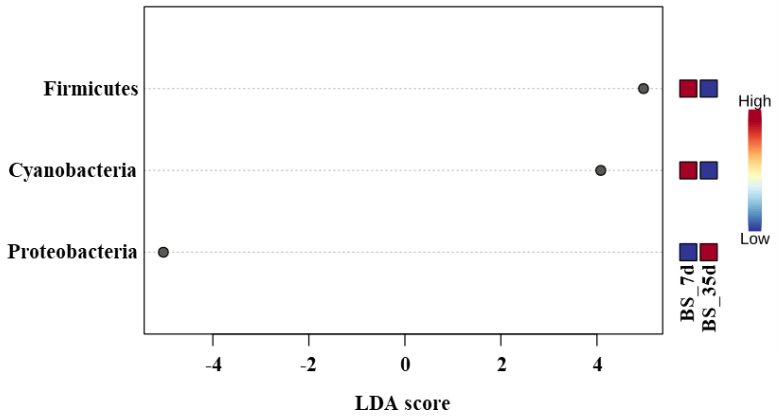


Figure 8. Profiles of cecal microbiota at the (A) phylum and (B) genus levels in broiler chickens treated with *Bacillus subtilis* on 7 and 35 days. The relative abundance of major phylum and genera in broiler chicken are illustrated. Genera accounting for < 0.5 % of all sequences across all 48 cecal samples are plotted as "Others". BS_7 days; 7-day-old broiler chickens from the group supplemented with *B. subtilis* and BS_35 days: 35-day-old broiler chickens from the group supplemented with *B. subtilis.*

A



B

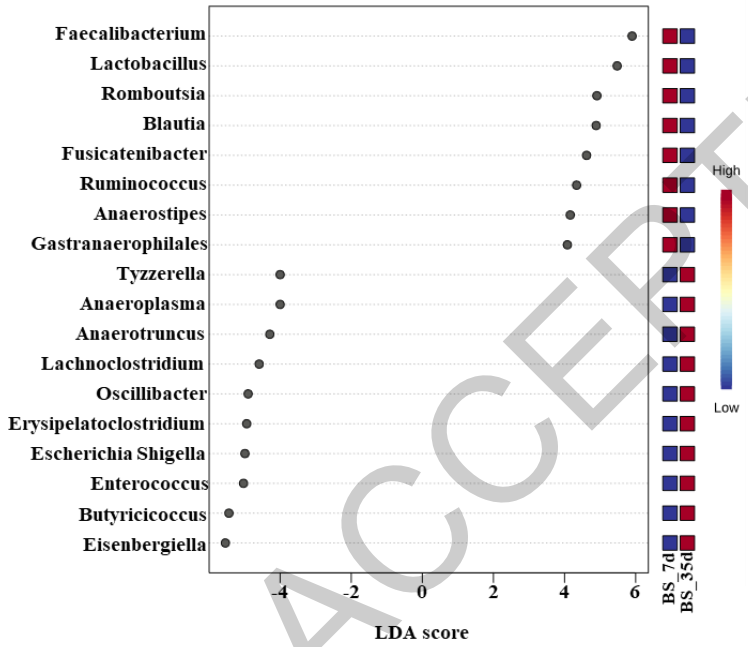


Figure 9. Differentially abundant (A) phyla and (B) genera in 7- and 35-day-old broiler chickens supplemented with *Bacillus subtilis*. Major phyla and genera that are differentially abundant with variations between age groups are supplemented with *B. subtilis* group and analyzed using the linear discriminant analysis (LDA) effect size (LEfSe) (LDA score > 4). BS_7 d: 7-day-old broiler chickens from the group supplemented with *B. subtilis* and BS_35 d: 35-day-old broiler chickens from the group supplemented with *B. subtilis.*