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Intestinal microbiota and transcriptomic alterations in the jejunum following supplementation with probiotic strain *Lacticaseibacillus paracasei* NSMJ15 in early-age broiler chickens

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Abstract

This study aimed to assess the effects of probiotic *Lacticaseibacillus* (*Lc*.) *paracasei* NSMJ15 supplementation on both the microbiota and transcriptome profiles in the small intestine of ten-days-old-broiler chickens. A total of 160 one-day-old male Ross 308 broiler chicks were randomly assigned to two dietary groups: a non-supplemented control group and an NSMJ15-supplemented group (8 pens per group, ten birds per pen). The diets consisted of a corn-soybean meal-based control diet and a test diet in which NSMJ15 (10^6 cfu/g) replaced 5 g/kg of ground corn. On day 10, one broiler per cage was euthanized, and jejunal contents and tissue samples were collected for microbiota and transcriptomic analyses. 16S rRNA amplicon sequencing demonstrated that NSMJ15 supplementation altered the bacterial community composition and increased bacterial diversity. Notably, the genus *Lacticaseibacillus* (*=Lc. paracasei* NSMJ15) reached an average relative abundance of 16.5% in the jejunum. However, the variation was observed among the samples, ranging from 0.16% to 51.79%, suggesting that the extent of NSMJ15 colonization may be influenced by the indigenous microbial community. To investigate the transcriptomic responses associated with NSMJ15 colonization, RNA-seq was performed on six selected samples (three control and three NSMJ15-supplemented). Mapping the RNA-seq reads to the chicken (*Gallus gallus*) genome revealed 287 differentially expressed genes (DEGs), including 140 upregulated and 147 downregulated genes. KEGG pathway enrichment analysis revealed that the cytokine-cytokine receptor interaction (CCRI) pathway was the most significantly enriched and several immune-related genes including *IL8, IL22*, *IFNL3A*, and *CCL4* were differentially expressed. In addition, indigenous genera such as *Mediterraneibacter*, *Blautia*, *Bacillus*, *Monoglobus*, *Lactococcus*, and *Pseudoescherichia* were altered in association with NSMJ15 colonization and showed significant correlations with host DEGs. Collectively, our data suggest that *Lc. paracasei* NSMJ15 supplementation may modulate jejunal microbiota composition and host immune- and nutrient metabolism-associated

gene expression, highlighting coordinated microbiota-host interactions in early-age broiler chickens.

Introduction

Poultry diseases result from intricate interactions among birds, their environments, and a range of infectious and non-infectious agents, which collectively impact overall health and productivity [1]. While antibiotics have historically been utilized to enhance growth and control disease [2], rising concerns about antimicrobial resistance and antibiotic residues in animal products have prompted regulatory bans on antibiotic growth promoters, most notably in the EU since 2006 [3-5]. As a consequence, interest in natural and safe alternatives such as probiotics, prebiotics, and postbiotics is growing in the pursuit of sustainable poultry production [6]. Probiotics, in particular, have demonstrated the ability to improve growth performance, support immune function, and enhance gut microbial equilibrium by inhibiting pathogen colonization through competitive exclusion [6,7].

Lactic acid bacteria (LAB) have a long history of safe application in humans and have also been employed for centuries in farm animal production as probiotics to support host health [8]. LAB are ubiquitous, occuring in varied environments such as dairy, meat, vegetables, soil, water, and in the gastrointestinal (GI) and the urogenital tracts of both humans and animals [9]. Their probiotic properties have been comprehensively investigated, focusing particularly on their roles in restoring intestinal microbial equilibrium, suppressing pathogenic organisms, and providing immunomodulatory and anti-inflammatory effects [10,11].

The small intestine (SI) is fundamental to mucosal immunity, functioning as a primary site of immune cell development and microbial interactions, particularly in the early life of broiler chickens. Commensal bacteria in the SI influence both local and systemic immune equilibrium by regulating T cell responses and cytokine secretion [12]. Therefore, characterizing microbial

populations and immune signaling within the SI is vital for enhancing host protection and maintaining intestinal health in poultry. Upon hatching, chicks experience a rapid colonization of the gastrointestinal tract by environmental microbes, which are critical for immune system development and establishing colonization resistance. Early exposure to microbes, such as through maternal feces or defined microbial consortia, has been demonstrated to improve immune parameters, including increased IgA and IgY levels, and to promote greater microbial diversity [13]. Due to the limited immune maturity of chicks at hatch, the prompt establishment of SI microbiota is necessary to stimulate proper mucosal immune function and provide lasting health advantages.

Various LAB strains exhibit strong abilites to colonize the small intestine (SI) of chickens following administration [14,15]. Such evidence supports the concept that probiotic LAB can act as early colonizers that interact with the host from initial developmental phases. Early establishment of a beneficial microbial community in the SI has been linked to functional improvements such as enhanced intestinal morphology and nutrient absorption [16], underscoring the value of focusing on the small intestinal niche in probiotic studies.

Lacticaseibacillus (*Lc*.) *paracasei* NSMJ15, a lactic acid bacterium (LAB) strain isolated from fermented products, has shown probiotic efficacy, such as antimicrobial action against intestinal pathogens [17,18]. Prior research indicated that the supplementation of NSMJ15 increases the ratio of CD4+ T cells in the small intestine and lowers acetate levels in cecal digesta by affecting the cecal microbiota of broiler chickens [19]. While individual immune system mediators such as cytokines have been widely researched [20], relatively little is known about the intricate interplay between the diverse elements that regulate immune activity. The current study aimed to thoroughly assess the impact of NSMJ15 supplementation on the small intestine (SI) of early-age broiler chickens by analyzing in the jejunal microbiota and transcriptome, and to clarify the interaction between probiotic LAB administration and host

responses. We hypothesized that NSMJ15 supplementation would alter the jejunal microbiota and be associated with changes in host intestinal transcriptomic responses, potentially contributing to intestinal function and host health in early-age broiler chickens.

Materials and Methods

Probiotic feed additive preparation

Lc. paracasei NSMJ15 was cultivated in de Man, Rogosa, and Sharpe (MRS) medium (BD Difco, Detroit, MI, USA) and harvested by centrifugation at 8,000 rpm for 20 min. The cell pellet was then resuspended in 10% (w/v) skim milk (MB cell, Seoul, Korea) as a protective medium, and subsequently lyophilized using a freeze dryer (FD-8512, Ilshin Lab Co., Ltd, Dongducheon, Korea) to yield a powdered product. The colony-forming units (CFU) per gram of powder were quantified using the standard plate-counting technique on MRS agar. Skim milk powder was incorporated to achieve a final concentration of 2×10^8 CFU/g of viable cells.

Animal experimental design and sample collection

One hundred sixty one-day-old male Ross 308 broiler chicks were randomly assigned to two treatments in a complete block design based on initial body weight. Each treatment consisted of eight replicates, with ten birds per replicate. The two experimental diets included a control diet based on corn-soybean meal and a diet supplemented with 5 g/kg *Lc. paracasei* NSMJ15 (10^6 cfu/g), replacing an equivalent amount of ground corn from the control formulation (Table S1). Both diets were formulated to satisfy the nutrient requirements recommended for Ross 308 broilers. Broilers were housed in wire-floored battery cages (60×50×60 cm) in an environmentally controlled facility under continuous lighting conditions. Feed and water were available ad libitum throughout the experiment period. All procedures and animal use protocols were approved by the Institutional Animal Care and Use Committee of Kyungpook National University (KU21083), Republic of Korea. On day 10 of the trial, one bird with median body

weight from each replicate was selected and euthanized using carbon dioxide asphyxiation. Jejunum samples were immediately collected following euthanasia. Digesta from the jejunum was sampled and stored at -20°C for subsequent metagenomic DNA extraction. An approximately 4-cm mid-segment from the mid-jejunum tissue was excised for total RNA extraction. Data related to growth performance were analyzed by one-way ANOVA utilizing the GLM procedure in SAS (SAS Inst. Inc., Cary, NC, USA). Dietary treatment and block were included as fixed effects in the statistical model. The experimental unit for growth performance analysis was the cage.

16S rRNA gene amplicon sequencing

For downstream microbiota analyses, eight control samples were included to provide balanced group sizes for comparison with the eight NSMJ15-treated samples. Genomic DNA from the jejunum digesta samples was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). The V3-V4 regions of the 16S rRNA gene were amplified with universal primers and Herculase II fusion DNA polymerase (Agilent Technologies, Santa Clara, CA, USA). The PCR protocol was as follows: (1) 3 min at 95°C; (2) 25 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C; (3) 5 min at 72°C. The universal primer set incorporating the Illumina adapter overhang sequence used for PCR was as follows: (1) V3-F: 5'-TCGTCGGCAGGTAGAGTAGAGTAGAGCCTACGGGGCWGCAG-3' and (2) V4-R: 5'-GTCTCGGGGGGGGGTAGAGAGAGAGAGAGAGAGACHVGTATATCC-3'. PCR products were purified using AMPure beads (Agencourt Bioscience, Beverly, MA, USA). The sequencing library was constructed with the Illumina 16S Metagenomic Sequencing Library protocol, and library quality was assessed using qPCR (KAPA Library Quantification kits for Illumina Sequencing platforms). Sequencing was conducted on the MiSeq™ platform (Illumina,

San Diego, USA) generating paired-end reads (2×300 bp) at Macrogen Inc. (Seoul, Republic of Korea).

Sequencing read processing and bacterial community analysis

Raw fastq data were assigned to individual samples using index sequences. Adapter and primer sequences were removed and paired-ends reads were produced, both using Cutadapt (v3.2). To generate amplicon sequence variants (ASVs), error-correction, denoising, and merging procedures were performed using DADA2 (v1.18.0) package in R. Quality filtering and correction for sequences with expected errors ≥2 were performed. Chimeric sequences were eliminated using the consensus method in DADA2. ASVs shorter than 350 bp were excluded and normalization was conducted using QIIME (v1.9) for microbial comparison analysis [21].

Taxonomic assignment of ASVs was conducted by BLAST+ (v2.9.0) against a reference database. Alpha diversity indices, including Chao1, Shannon, and Inverse Simpson were calculated in QIIME (v1.9) utilizing ASV abundance and taxonomic information. Principal-component analysis (PCA) was performed to assess bacterial community variation among jejunum samples, based on relative abundance of all taxonomic groups, using the Vegan package in R. For identification of taxa differing after probiotics treatment, linear discriminant analysis (LDA) effect size (LEfSe) was performed [22]. The alpha value for the factorial Kruskal-Wallis test was set to 0.05 and the LDA score threshold was 2.0.

Bulk mRNA sequencing

For transcriptomic analysis, control samples dominated by non-LAB and NSMJ15-supplemented samples with high *Lacticaseibacillus* abundance were selected to investigate transcriptomic changes associated with NSMJ15 colonization. Each jejunum sample (20 mg) was combined with 750 μl of QIAzol® Lysis Reagent (QIAGEN, Germany) in a tube containing a stainless steel bead and homogenized with a tissuelyser (QIAGEN) at 20 Hz for 2 min.

Subsequently, 150 µl of chloroform was added to the homogenized sample, vortexed for 15 sec, and incubated at room temperature for 2 min. After centrifugation at 12,000 g for 15 min, the upper aqueous phase was transferred to a new tube. Following this, 350 µl of RLT buffer (RNeasy Mini Kit; QIAGEN) and 250 µl absolute ethanol were then added to the aqueous phase and mixed gently. The mixture was loaded onto an RNeasy spin column (RNeasy Mini Kit; QIAGEN) and filtered using a vacuum manifold. The column-bound RNA was washed once with 700 µl Buffer RWT. A total of 80 µl DNase I was applied directly onto the column and left at room temperature for 5 min. The RNA sample was subsequently washed twice with 500 µl Buffer RPE (Qiagen), subjected to a dry spin without buffer, and eluted with 50 µl RNase-free water. The purified RNA was stored at -80°C.

The concentration of the extracted RNA was determined using Quant-IT RiboGreen (Invitrogen, Carlsbad, CA, USA). RNA integrity was evaluated with the TapeStation RNA ScreenTape (Agilent Technologies, Santa Clara, CA, USA). High-quality RNA, defined as samples with an RNA integrity number (RIN) greater than 7.0, was selected for RNA library construction. Total RNA (0.5 µg) was utilized to prepare the library using the Illumina TruSeq Stranded Total RNA Library Prep Gold Kit (Illumina). Briefly, rRNA was selectively removed from each RNA sample utilizing the Ribo-Zero rRNA Removal Kit (Illumina). After purification, the mRNA was fragmented into smaller pieces by exposure to divalent cations at elevated temperature. Subsequently, the fragmented mRNA was reverse transcribed to synthesize first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen) and random primers. Second-strand cDNA was then generated using DNA Polymerase I, RNase H, and dUTP. The resulting cDNA fragments underwent end repair, an 'A' base addition, and ligation with sequencing adapters. The adapter-ligated products were purified and PCR-amplified to produce the final cDNA libraries. Library concentrations were measured by qPCR using the KAPA Library Quantification kit for Illumina platforms (KAPA Biosystems,

Wilmington, MA, USA), and their quality was verified with the TapeStation D1000 ScreenTape (Agilent Technologies). Paired-end sequencing (2x100 bp) was performed on the Illumina NovaSeq platform (Illumina) at Macrogen Inc. (Seoul, Republic of Korea).

Data processing and analysis

Trimmomatic (v0.38) [23] was employeed to remove adapter sequences and low-quality bases. The cleaned reads were subsequently aligned to the *Gallus gallus* (Chicken) GRCg7b reference genome using HISAT v2.1.0 [24]. The reference genome and gene annotation files were sourced from the NCBI Genome Assembly and NCBI RefSeq databases, respectively. The sequence alignment mapping (SAM) files were sorted and indexed using SAMtools v1.9 [25]. Following alignment, transcript assembly and abundance estimation were conducted with StringTie v2.1.3b [26,27]. Quantification at both gene and transcript levels was performed as raw read count, FPKM (Fragments Per Kilobase of transcript per Million mapped reads), and TPM (Transcripts Per Million).

Differentially expressed gene (DEG) analysis

Statistical analyses for differential gene expression were conducted using DESeq2 (v 1.38.3) [28] with raw counts provided as input. During the QC step, only genes expressed (non-zero counts) in all samples were analyzed. To assess expression similarity among samples, MDS (multi-dimensional scaling) analysis was performed. RNA-seq read counts were normalized using RLE (Relative Log Expression) to correct for differences in library sizes. Differential expression was evaluated using the negative binomial Wald test in DESeq2. Fold changes and raw p-values were obtained from the Wald test, and genes with a fold change (FC) of ≥ 2 or ≤ -2 and a raw p-value ≤ 0.05 were defined as differentially expressed genes (DEGs).

Gene-enrichment and functional annotation analysis of the DEGs was carried out using g:Profiler [29] against the GO (Gene Ontology) database, as well as an in-house KEGG (Kyoto

Encyclopedia of Genes and Genomes) Viewer script for analyzing the KEGG pathway database (http://www.genome.jp/kegg/pathway.html). GO enrichment significance was evaluated using Benjamini–Hochberg-adjusted p-values, whereas KEGG pathway enrichment was evaluated using a modified Fisher's exact test with p-value ≤ 0.05.

DEGs were mapped to the search tool for retrieval of interacting genes (STRING) database (https://string-db.org/) [30] to obtain protein–protein interaction (PPI) networks. The species parameter was set to "*Gallus gallus*" with a default confidence threshold of 0.4.

Network analysis between bacterial abundance and host gene expression

To investigate interactions between host DEGs and differentially altered bacterial genera following probiotic NSMJ15 supplementation, Spearman correlation coefficients were determined using the *cor.test* function in RStudio (version 3.5.0). For the correlation analysis, bacterial relative abundance and TPM values of DEGs derived from alignment to the *Gallus gallus* (Chicken) GRCg7b genome and the KEGG pathway database were utilized. Correlations with a p-value ≤ 0.05 were considered statistically significant for bacteria-host gene expression associations. Network visualizations were constructed with Cytoscape v3.10.2 [31].

Data deposition

The raw sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under accession numbers PRJNA1400232 for the 16S rRNA gene amplicon sequencing and transcriptomic data.

Results

Growth performance of broiler chicks fed experimental diets

Table S2 presents the effects of *Lc. paracasei* NSMJ15 supplementation on the growth metrics of 10-day-old broilers maintained on either a non-supplemented control or an NSMJ15-

supplemented diets. No statistically significant differences were detected in body weight (BW) gain, feed intake (FI), and the feed efficiency ratio (G:F) between birds receiving two diets.

Alteration of jejunal microbiota of broiler chicks fed a diet with *Lc. paracasei* NSMJ15 supplementation

Using the Illumina Miseq platfom, 3,687,530 bacterial 16S rRNA gene sequencing reads were initially generated. After filtering for quality and eliminating chimeric sequences, a total of 1,601,415 reads (43.4% of the original) were retained for analysis of jejunal microbiota in ten-days-old broiler chickens fed *Lc. paracasei* NSMJ15-supplemented diets (Table S3). Bacterial diversity and richness indices calculated for non-supplemented control group (NS) and NSMJ15-supplemented group (NSMJ15-S) revealed increased richness (Chao1) and Shannon index values in the NSMJ15-S samples compared to the NS group (Fig. 1A). Comprehensive diversity and richness metrics, including ASV2, Chao1, Shannon index (H'), and Gini-Simpson index, confirmed the shifts in bacterial diversity observed in all examined samples (Table S3).

The bacterial 16S rRNA gene sequencing reads were classified at the genus level to assess alterations in the microbiota resulting from *Lc. paracasei* NSMJ15 supplementation (Fig. 1B). Several taxa displayed differential abundance when comparing the NS group with the NSMJ15-S group. The NSMJ15-S group exhibited, on average, a 21.0% higher relative abundance of the lactic acid bacteria (LAB) group than the NS group (NS: 51.6%, NSMJ15-S: 72.6%) (Fig. 1B). Notably, a significant elevation in the genus *Lacticaseibacillus* was detected in the NSMJ15-S group (NS: 0.03%, NSMJ15-S: 16.5%) (Fig. 1B). Linear discriminant analysis effect size (LEfSe) analysis highlighted genera showing significant enrichment in the NSMJ15-S group (Fig. 1C). In addition to the observed increase in the genus *Lacticaseibacillus*, the low-abundant genus, *Monoglobus*, also showed increased abundance following NSMJ15 supplementation. Conversely, the low-abundant genus, *Pelomonas* was more prevalent in the NS group.

Variation in the microbiota composition and its response to *Lc. paracasei* NSMJ15 supplementation in the jejunum of broiler chicks

The microbiota composition analysis revealed variation in the indegenous microbiota structure, as well as the microbial alterations after *Lc. paracasei* NSMJ15 supplementation in the jejunum of broiler chickens (Fig. 2). Notably, the relative abundance of lactic acid bacteria group (LAB) among the non-supplemented control group (NS) varied widely from 2.9% to 99.9%, with an average of 49.7% (Figs 2A and B). The genus *Lactobacillus* predominated in NS2, NS3, and NS7, while both *Lactobacillus* and *Ligilactobacillus* were dominant in NS5. Among the non-LAB genera, *Pseudoescherichia* was most prevalent in NS1, NS6, and NS8, whereas *Clostridioides* dominated in NS4 (Figs 2A). After ten days of NSMJ15 supplementation, the relative abundance of LAB ranged from 27.9% to 99.3%, with an average of 64.5% (Figs 2A and B). The relative abundance of the genus *Lacticaseibacillus* increased to varying extents among the NSMJ15-supplemented group (NSMJ15-S) (Fig. 2A). Notably, *Lacticaseibacillus* was highly abundant in NSMJ15-S1 (51.8%), -S2 (29.5%), and -S3 (38.5%), whereas lower levels were observed in S4 through S8 (0.2% to 5.4%). In the latter samples, other LAB genera including *Lactobacillus*, *Limosilactobacillus*, and *Ligilactobacillus* were more abundant than *Lacticaseibacillus*.

In addition to microbial composition results, principal component analysis (PCA) supported the variation in the indegenous microbiota structure and the impact of *Lc. paracasei* NSMJ15 supplementation in the jejunum (Fig. 2B). In the PCA, NS2, NS3, and NS7 formed a single cluster, with NS1, NS6, and NS8 grouped separately. NS4 and NS5 also showed distinct microbial profiles. The NSMJ15-S1, -S2, and -S3 displayed clear shifts in community composition, characterized by an increased proportion of *Lacticaseibacillus* relative to NS. In contrast, the remaining NSMJ15-supplemented samples (S4 to S8) clustered more closely with the NS, suggesting a less substantial response to NSMJ15 supplementation.

Differential gene expression in the jejunum of broiler chicks fed a diet supplemented with *Lc. paracasei* NSMJ15

To evaluate transcriptomic alterations in the jejunum following colonization of *Lc. paracasei* NSMJ15, three non-supplemented control samples (NS1, NS6, and NS8) dominated by non-LAB and three NSMJ15-supplemented samples (NSMJ15-S1, -S2, and -S3) exhibiting high abundance of the genus *Lacticaseibacillus* were selected for the bulk mRNA sequencing (Fig. 2). The NS samples dominated by non-LAB were chosen to minimize interference from endogenous LAB, thereby enabling clearer detection of NSMJ15-associated transcriptomic responses. cDNAs synthesized from purified mRNA were subjected to sequencing, generating a total of approximately 434.1 million reads (60.7–80.5 million reads per sample) (Table S4). Reads of low quality and shorter than 50 bp were filtered out, resulting in clean reads constituting 94.1–98.1% of the total (Table S4). Between 94.3% and 97.0% of the clean reads mapped to the *Gallus gallus* GRCg7b reference genome.

Multidimensional scaling (MDS) and hierarchical clustering analysis of gene expression showed clear separation between three NS and three NSMJ15-S samples (Fig. 3). Differentially expressed genes (DEGs) analysis confirmed shifts in jejunal transcriptomic profiles following *Lc. paracasei* NSMJ15 supplementation; 287 DEGs (absolute fold change (FC) of ≥ 2 and raw p-value ≤ 0.05) were detected (Fig. 4). Among these, 140 genes were identified as up-regulated and 147 as down-regulated in the NSMJ15-S compared with the NS samples. The lists of up-regulated and down-regulated genes, along with their fold-change (FC) and p-values, are provided in Table S5. The results indicated *SLC5A12* ($\log_2$FC=4.10), *GJA3* (4.03), *CMD4* (3.29), *GABRG3* (3.10), LOC121108166 (3.06), LOC121108164 (2.65), *ADH1C* (2.63), *ART1L3* (2.55), *CALB1* (2.51), and LOC121108134 (2.43) as the top ten up-regulated genes in the jejunum. In contrast, *SAA* ($\log_2$FC=-6.03), *EXFABP* (-5.98), *SERPINB10B* (-4.91), *AVD* (-4.79), *IL22RA2* (-3.89), *ACOD1L* (-3.74), LOC107057061 (-3.71), *NOXO1* (-3.34), *MMP9* (-

3.33), and *LYG2* (-3.32) were the ten most down-regulated genes in the NSMJ15-supplemented chickens.

Functional annotation of differentially expressed genes in the jejunum of broiler chicks receiving *Lc. paracasei* NSMJ15 dietary supplementation

To characterize the functional properties of differentially expressed genes (DEGs) and identify biological pathways potentially altered by *Lc. paracasei* NSMJ15 supplementation, GO and KEGG pathway enrichment analyses were performed. The list of differentially expressed genes (DEGs) in the jejunum was examined according to the three categories in Gene Ontology (GO): biological process (BP), cellular component (CC), and molecular function (MF). Multiple overrepresented BP, CC, and MF terms were observed in the jejunum (adjusted p-value ≤ 0.05) (Fig. S1). Notably, BP terms including cellular response to chemical stimulus, response to external stimulus, transmembrane transport, and response to bacterium, as well as CC terms such as extracellular region and extracellular space, were enriched in the jejunum of ten-day-old broiler chickens following *Lc. paracasei* NSMJ15 supplementation. To further characterize the functional consequences of NSMJ15 supplementation, KEGG pathway enrichment analysis was performed. A total of 41 DEGs were mapped to fourteen significantly enriched pathways in the jejunum (p-value ≤ 0.05) (Fig. 5A). The three most significantly enriched pathways were cytokine–cytokine receptor interaction, neuroactive ligand–receptor interaction, and alanine, aspartate, and glutamate metabolism. Other pathways that were significantly enriched included neuroactive ligand signaling, arginine biosynthesis, vitamin digestion and absorption, tyrosine metabolism, tryptophan metabolism, insulin signaling pathway, retinol metabolism, influenza A, toll-like receptor signaling pathway, phenylalanine metabolism, and glycerophospholipid metabolism.

Gene network analysis of differentially expressed genes in broiler chicks following *Lc. paracasei* NSMJ15 supplementation

To further evaluate interactions among the 41 DEGs (27 downregulated and 14 upregulated) associated with the enriched KEGG pathways, a protein-protein interaction (PPI) network was constructed using interaction data from the STRING database (Fig. 5B). Genes involved in the cytokine–cytokine receptor interaction (CCRI) pathway formed a connected cluster, including key cytokine- and chemokine-related genes such as *IL22*, *IFNL3A*, *CCL4*, and *IL8L1*/*2*, which were predominantly downregulated following NSMJ15 supplementation. Genes involved in amino acid biosynthesis and metabolism, including *ASL*, *ASL1*, *ASS1*, *IL4l1*, *DDC*, and *HAAO*, formed another cluster linked to the CCRI pathway. These genes were also predominantly downregulated, with the exception of *DDC*. In addition, two minor clusters (*GNG4-DRD1-TAC1-CHAT* and *C5*-LOC428593) associated with neuroactive ligand signaling were identified, containing both upregulated and downregulated genes. Three additional minor clusters (*SCARB1-LCAT*, *G6PC-GYS2*, and *ADH1C-ALDH1A2-ETNPPL*) were associated with nutrient metabolism-related pathways, and genes within these clusters were mostly upregulated. Collectively, these data suggest coordinated interactions between immune and metabolic processes in the jejunum following *Lc*. *paracasei* NSMJ15 supplementation.

Correlation network analysis between the microbial community and DEGs in the jejunum of broiler chicks fed a diet with *Lc*. *paracasei* NSMJ15

To explore possible interactions between the gut microbiota and gene expression in the jejunum, Spearman correlation analysis was performed to construct a correlation network. Additional LEfSe analysis of microbiota data from the six samples used for RNA-seq analysis identified seven significantly altered genera, including six increased genera (*Lacticaseibacillus*, *Mediterraneibacter*, *Blautia*, *Bacillus*, *Monoglobus*, and *Lactococcus*) and one decreased genus

(*Pseudoescherichia*), which were incorporated into the network analysis (Fig. 6A). Correlation analysis between these genera and the 41 DEGs associated with enriched KEGG pathways revealed significant associations with a total of 27 DEGs (Fig. 6B). Among the seven altered genera, *Lacticaseibacillus* showed correlations with multiple DEGs, including negative associations with cytokine–cytokine receptor interaction (CCRI)-related genes (*IL8L1* and *IL20RA*), neuroactive signaling-related genes (*TAAR1*, *PTAFR*, and *GPR83L*), and amino acid metabolism-related genes (*ASL* and *ASL1*), as well as positive associations with genes related to neuroactive signaling (*C5* and *S1PR2*) and vitamin digestion and absorption (*SLC19A2*). *Lactococcus*, *Blautia*, *Mediterraneibacter*, *Monoglobus*, and *Bacillus* showed similar correlation patterns with several DEGs, including *CCL4*, *SOCS1*, *GYS2*, *CUBN*, *LCAT*, *TAC1*, *GABRG3*, and *ETNPPL*. In contrast, *Pseudescherichia* displayed opposite correlation patterns relative to these genera. Collectively, these results suggest coordinated associations between altered gut microbiota and host immune- and metabolic process in the jejunum following *Lc. paracasei* NSMJ15 supplementation.

Discussion

The small intestine serves not only as the primary site of nutrient digestion and absorption but also as a critical interface for early immune development in poultry [32]. During the post-hatch period, interactions between colonizing microbiota and the developing intestinal immune system contribute to the establishment of mucosal immunity and long-term immune competence [33-35]. Therefore, modulation of the jejunal microbiota through early probiotic intervention may influence both microbial community development and host immune responses. Previous studies have shown that probiotics can promote microbial diversity and increase the abundance of beneficial bacteria in the small intestine, thereby supporting intestinal health and immune function [36,37].

In an earlier investigation, dietary supplementation with the probiotic strain *Lc*. *paracasei* NSMJ15 influenced immune cell populations and altered microbial community structure in broiler chickens [19]. However, that study primarily focused on immune cell profiles in the jejunum and microbial changes in the cecum, without examining functional gene expression or detailed microbial dynamics within the small intestine during early development. In the present study, we further investigated the effects of NSMJ15 supplementation on both jejunal microbiota composition and host transcriptomic responses in neonatal broiler chickens, providing new insights into microbiota-associated regulation of immune- and metabolism-related pathways in the small intestine.

In this investigation, supplementing the diets of neonatal broiler chickens with *Lc*. *paracasei* NSMJ15 resulted in intestinal colonization with an average relative abundance of 16.5%, accompanied by increased microbial diversity as measured by Shannon and Chao1 indices, along with changes of jejunal microbiota composition (Fig. 1). These modifications could affect gut microbiota and immune system maturation in early life. Along with the increase in *Lc*. *paracasei*, Fig. 1C highlighted an increased abundance of *Monoglobus*, a genus associated with pectin degradation and intestinal carbohydrate fermentation [38]. These microbial processes contribute to nutrient utilization and gut metabolic activity, potentially influencing host intestinal physiology and microbiota-associated responses [39]. One decreased genus, *Pelomonas*, has previously been reported as environmental bacterium [40]; however, its functional role in the gut remains poorly understood.

The qualitative and quantitative characteristics of intestinal changes may vary depending on the probiotic strain, dosage, and its prevalence within the intestinal microbiota. The present study indicated that these differences could also be influenced by the indigenous microbial composition of the small intestine, leading to individual variability (Fig. 2). Notably, the dominance of the administered probiotic *Lc*. *paracasei* NSMJ15 (0.2% to 51.8%) differed

significantly among individuals, which appeared to be linked to the distinct indigenous microbial communities present in each subject (Fig. 2). In non-supplemented control group, four individuals (NS1, 4, 6, and 8) exhibited a predominance (84.5% to 97.6%) of non-lactic acid bacteria group (non-LAB), including *Pseudoescherichia*, *Clostridioides*, *Trichocoleus*, *Enterococcus*, *Mediterraneibacter*, *Prauserella*, and *Blautia* (Figs 2A and C). Conversely, LAB group including *Lactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, and *Lactiplantibacillus* were predominant (83.0% to 99.9%) in the other four individuals (NS2, 3, 5, and 7). In the NSMJ15-supplemented group, five individuals (S4-S8) with a greater abundance of LAB (especially *Lactobacillus* spp.) showed reduced dominance (0.2% to 5.4%) of the *Lc*. *paracasei* NSMJ15, while those (S1-S3) with higher amounts of non-LAB demonstrated relatively greater dominance (29.5% to 51.8%) of the probiotic NSMJ15 (Figs 2A and B). These findings suggest that *Lc*. *paracasei* NSMJ15 may interact with both LAB and non-LAB during colonization in the small intestine, resulting in differences in colonization levels among individuals. The strain NSMJ15 has been reported to exhibit antimicrobial activity against pathogenic bacteria, including *Escherichia coli* and *Staphylococcus aureus* [18], highlighting its potential to influence the intestinal microbial community. These observations indicate that NSMJ15 supplementation may not induce uniform effects across all individuals, but rather result in responder-specific microbiota-driven responses depending on the initial microbial community structure. Collectively, these findings suggest that interactions between the administered probiotic and indigenous microbial communities may influence colonization success and host responses, although the underlying mechanisms remain to be experimentally validated. A better understanding of these mechanisms may contribute to the development of more effective probiotic strategies in poultry.

The substitution of dominant non-LAB with the probiotic *Lc*. *paracasei* NSMJ15 and the corresponding rise in LAB populations are anticipated to enhance intestinal development and

support host health (Figs 2A and B). Previous studies have shown that probiotic supplementation increases LAB abundance, suppresses pathogen colonization, and positively effects intestinal morphology [41]. Additionally, it has been demonstrated to strengthen intestinal barrier integrity through the upregulation of tight junction proteins (ZO-1, Claudin-1, and Occludin) and to mitigate intestinal damage induced by *Salmonella Enteritidis* [42]. *Lactobacillus acidophilus* has shown immunomodulatory effects, enhancing serum immunoglobulins (IgA, IgG, and IgM) and promoting lymphocyte proliferation [43]. Furthermore, strains of *Lactobacillaceae* isolated from chicken caeca have demonstrated protective effects against *Clostridium perfringens*-induced necrotic enteritis, highlighting their role in enhancing immune responses and resisting pathogen colonization [44]. Together, the modifications in the gut microbiota mediated by LAB may facilitate immune homeostasis, improve intestinal integrity, and increase resistance to enteric pathogens in broiler chickens.

Although the sample size was limited and the transcriptomic analysis was performed using birds selected based on their NSMJ15 colonization levels, the samples exhibited distinguishable jejunal transcriptomic profiles compared with the control samples dominated by non-LAB (Fig. 3). The findings may not fully represent the transcriptomic responses of the entire treated population. Our transcriptomic assessment identified genes and associated functions in the intestinal tissue that are regulated by NSMJ15 (Figs 4 to 6 and Table S5). Notably, the modulation of cytokine-cytokine receptor interaction (CCRI) pathway following the NSMJ15 supplementation could be an indicative of its probiotic-mediated immunomodulatory properties (Fig. 5). In particular, the downregulation of inflammation-related interleukin and chemokine genes such as *IL8L1/2* and *CCL4* within the CCRI pathway supports the possibility, which reduces intestinal immune activation by diminishing the expression of pro-inflammatory mediators. IL8 and CCL4 are linked to both acute and chronic inflammatory reactions in immune cells, with elevated expression reported during various biological infections and

abiotic stress in poultry [45,46]. In addition, reduced expression of *SAA*, *IL22*, and *IFNL3A* following the NSMJ15 supplementation may correspond to decreased mucosal immune activation, potentially indicating the establishment of a more stable gut environment with minimal inflammatory or pathogenic stimuli. Nevertheless, alternative explanations should be considered, as the observed reduction in immune activation may be influenced by developmental stage-dependent processes and indirect regulation mediated by changes in the gut microbiota. The upregulation of genes involved in nutrient absorption and metabolism, including *CALB1* (calcium transport) [47], *SLC5A12* (sodium-coupled monocarboxylate transport) [48], and *ASS1* and *ASL* (arginine biosynthesis and metabolism) [49], together with genes associated with immune homeostasis and cytokine signaling regulation (*SOCS1*) [50], and neuroimmune interactions (*GABRG3*) [51], suggests that NSMJ15 supplementation may influence multiple aspects of intestinal physiology, including nutrient utilization and immune regulation (Figs 4, 5, and Table S5).

These observed alterations in jejunal gene expression may have resulted from the direct effects of *Lc. paracasei* NSMJ15 or indirect effects mediated through changes in the intestinal microbiota (Fig. 6). LEfSe analysis of the samples selected for transcriptomic analysis revealed increased abundance of *Mediterraneibacter*, *Blautia*, *Bacillus*, *Monoglobus*, and *Lactococcus*, along with a decreased abundance of *Pseudoescherichia* in the NSMJ15-supplemented group compared with the control. Several of the enriched genera have previously been associated with beneficial gut functions. For example, *Bacillus* and *Lactococcus* are recognized for their probiotic potential [52, 53], whereas *Monoglobus* has been linked to pectin degradation and intestinal carbohydrate fermentation, which may contribute to nutrient utilization and gut metabolic activity [38]. In contrast, although genus *Pseudoescherichia* was reduced following NSMJ15 supplementation, its role in the chicken intestine remains poorly understood. Nevertheless, some members of this genus, including *Pseudoescherichia vulneris* (previously

Escherichia vulneris), have been reported as opportunistic pathogens in human and animals [54]. These microbial shifts may have contributed to the transcriptomic alterations observed in the jejunal intestinal tissue, as further supported by the microbiota-gene correlation network analysis (Fig. 6A). The correlation network analysis revealed potential of distinct microbiota–host associations, with *IL8L1* being primarily correlated with *Lacticaseibacillus*, whereas *CCL4* and *SOCS1* were correlated with multiple altered genera, including *Mediterraneibacter*, *Blautia*, *Bacillus*, *Monoglobus*, *Lactococcus*, and *Pseudoescherichia*. These findings suggest that host immune responses may be shaped by both specific bacterial taxa and broader microbial community shifts (Fig. 6B). In addition to immune-related genes, several genes involved in nutrient metabolism (*ADH1C*, *SLC19A2*, *CUBN*, *ASS1*, and *ASL*) and neuroimmune signaling (*GABRG3*) were correlated with the altered bacterial genera. Therefore, the observed transcriptomic alterations may reflect not only the direct effects of NSMJ15 colonization but also synergistic interactions between NSMJ15 and the reshaped intestinal microbial community.

Conclusion

Our data shows that colonization by *Lc*. *paracasei* NSMJ15 was influenced by the composition of the indigenous jejunal microbiota, resulting in responder-specific microbial and transcriptomic alterations in newborn broiler chickens. Colonization by *Lc*. *paracasei* NSMJ15 was associated with modulation of immune-related genes, including *IL8*, *IL22*, *IFNL3A*, and *CCL4*, together with genes involved in nutrient absorption and metabolism, suggesting a potential role in shaping host immune and metabolic responses in the jejunum. In addition, several indigenous microbial taxa including genera *Mediterraneibacter*, *Blautia*, *Bacillus*, *Monoglobus*, *Lactococcus*, and *Pseudoescherichia* also increased with colonization of NSMJ15, and these alterations were correlated with shifts in jejunal gene expression. These findings provide preliminary evidence that *Lc*. *paracasei* NSMJ15 may contribute to microbiota-

associated transcriptomic modulation related to host health, and further studies, including phenotypic validation, challenge models, and analyses in broader populations, are required to validate its functional effects and potential as an antibiotic alternative in poultry production. In addition, metagenomic assessments of antibiotic resistance gene profiles could provide further insight into the safety of NSMJ15 and its potential as a probiotic alternative to antibiotic growth promoter.

Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions

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Ethics approval and consent to participate

All procedures and animal use protocols were approved by the Institutional Animal Care and Use Committee of Kyungpook National University (approval number: KU21083).

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FIGURE LEGENDS

Fig. 1. (A) Chao1 and Shannon diversity indices assessing bacterial richness and diversity, and (B) microbiota composition (%) in the jejunum of control (NS) and *Lc. paracasei* NSMJ15-supplemented (NSMJ15-S) groups. Genera with relative abundance more than 0.5% of the total reads in respective samples are displayed. (C) LEfSe analysis showing the most differentially abundant taxa between the two groups. LDA scores are negative for the control group and positive for the *Lc. paracasei* NSMJ15-supplemented group.

Fig. 2. Composition (%) of (A) overall microbiota and (B) lactic acid bacteria (LAB) group in the individual samples from control (NS) and *Lc. paracasei* NSMJ15-supplemented (NSMJ15-S) groups. Genera accounting for more than 0.5% of the total reads in respective samples are presented. (C) Principal-component analysis (PCA) score plot of the jejunal microbiota illustrating a comparison between samples from the control and *Lc. paracasei* NSMJ15-supplemented groups.

Fig. 3. (A) A multidimensional scaling (MDS) plots and (B) hierarchical clustering of differentially expressed genes (DEGs) in the jejunal tissue of control (NS) and *Lc. paracasei* NSMJ15-supplemented (NSMJ15-S) groups. DEGs were defined as those with a raw p-value ≤ 0.05 and an absolute fold-change ≥ 2 between groups.

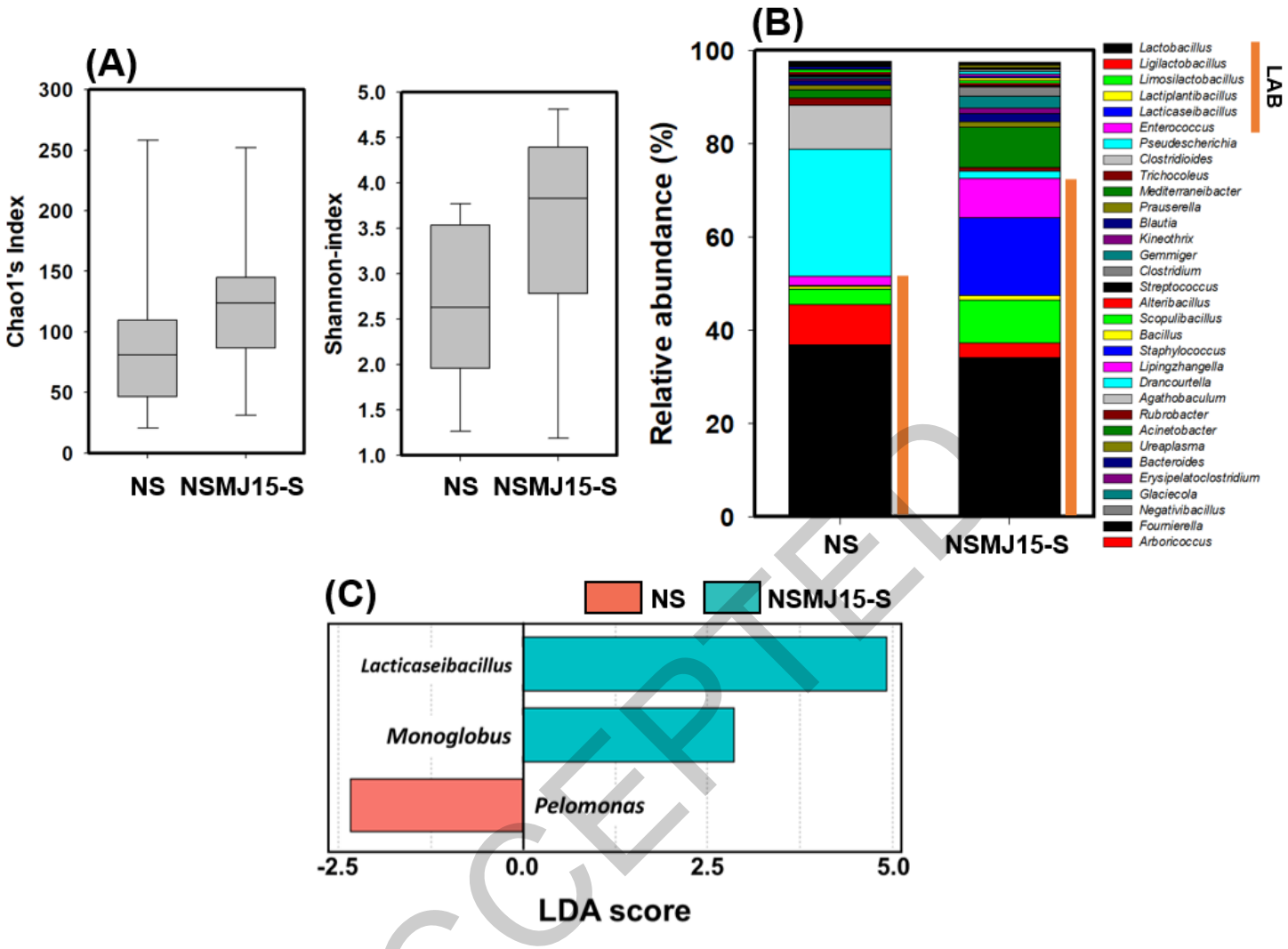
Fig. 4. Volcano plots illustrating differentially expressed genes (DEGs) in the jejunal tissue of ten-days-old broiler chickens following *Lc. paracasei* NSMJ15 supplementation. DEGs were defined as genes with a raw p-value ≤ 0.05 and an absolute fold-change ≥ 2. Representative down-regulated and up-regulated genes are indicated.

Fig. 5. (A) KEGG pathway enrichment analysis of DEGs in the jejunal tissue of ten-days-old broiler chickens following *Lc. paracasei* NSMJ15 supplementation. Significantly enriched

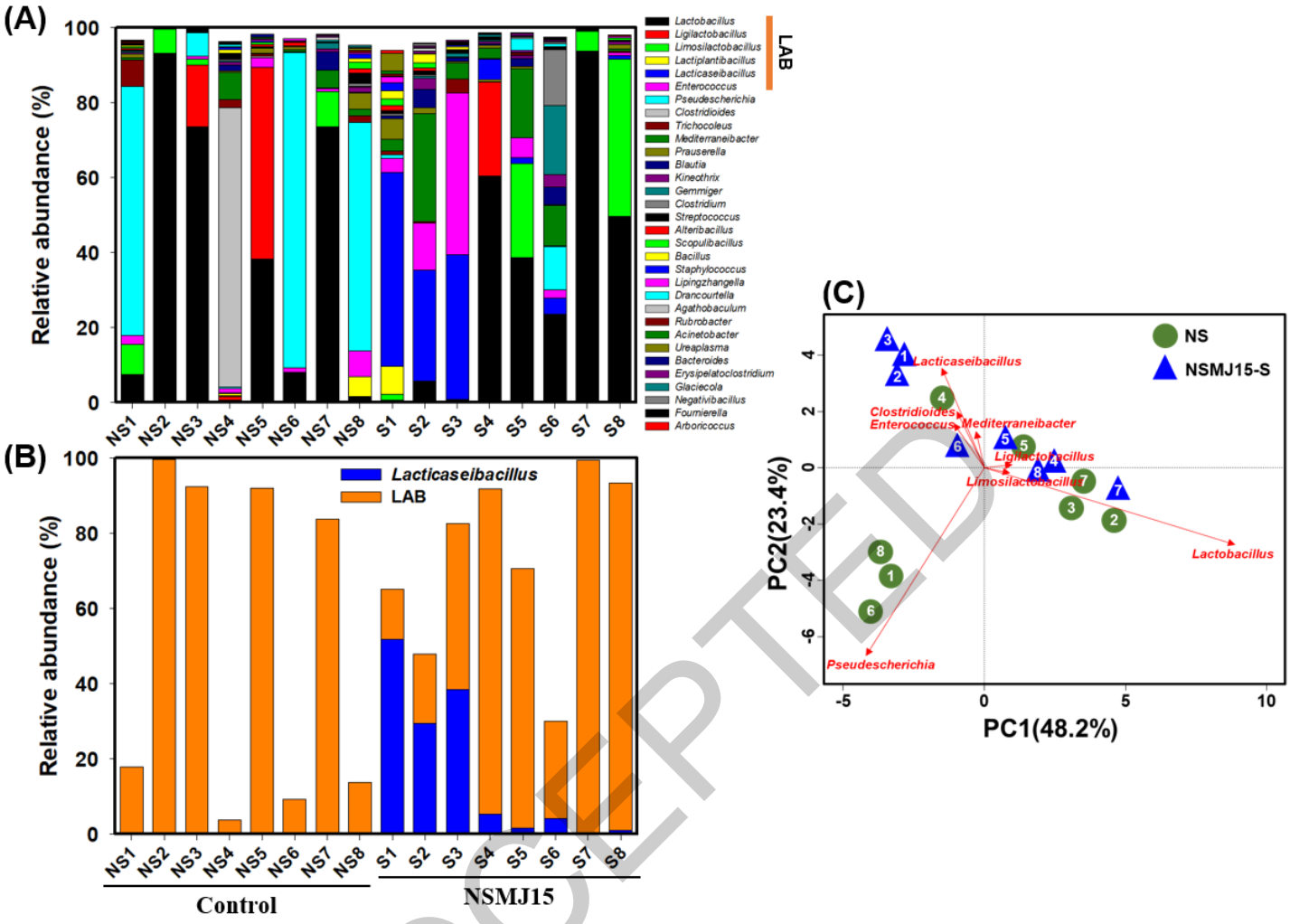
pathways (p-value ≤ 0.05) were identified using a modified Fisher's exact test. The x-axis represents the –log(p-value) for each pathway. (B) Protein-protein interaction (PPI) network analysis of DEGs mapped to enriched KEGG pathways. Blue upward arrows indicate upregulated genes, whereas red downward arrows indicate downregulated genes. The species parameter was set to "*Gallus gallus*" with a default confidence threshold of 0.4.

Fig. 6. (A) LEfSe analysis showing the most differentially abundant taxa between control (NS) and *Lc*. *paracasei* NSMJ15-supplemented (NSMJ15-S) groups from the six selected samples. (B) Integrated correlation-based network analysis illustrated interactions between microbes and DEGs. Spearman correlation evaluated the relationships with solid and dotted lines representing positive and negative correlations, respectively.

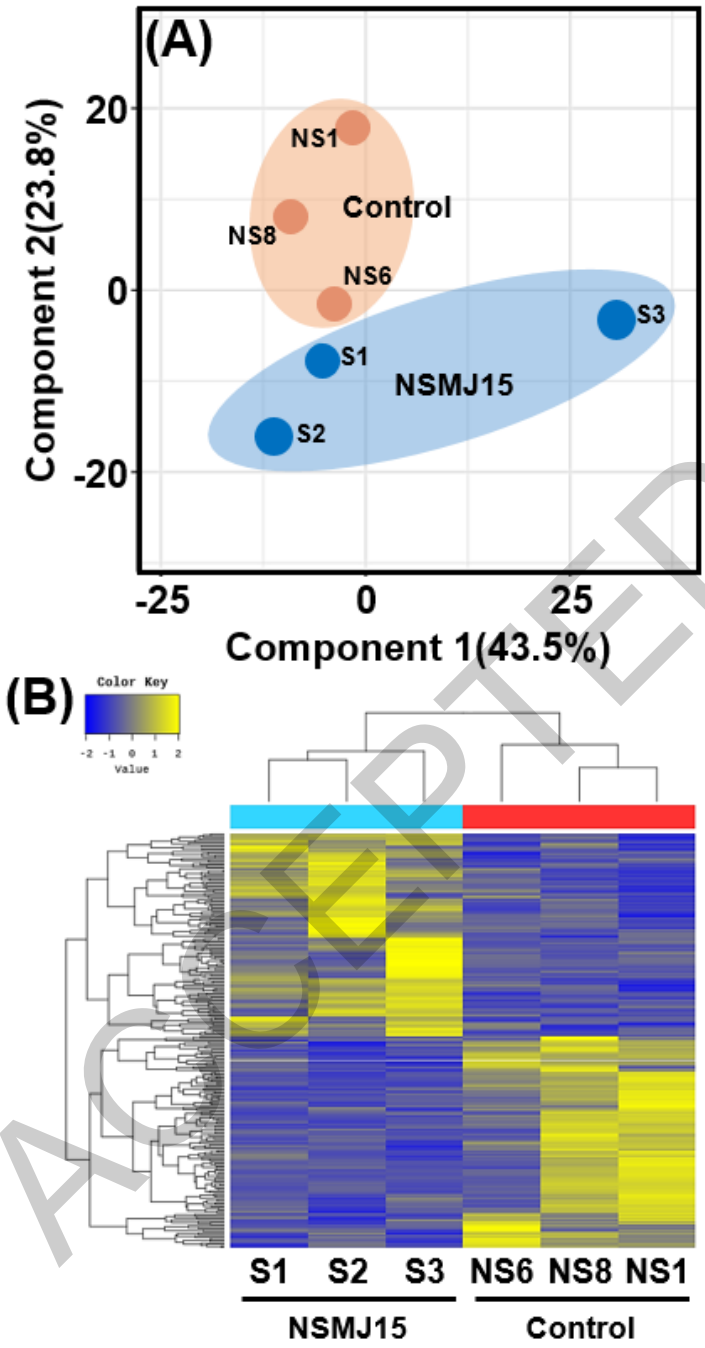
737 **Fig. 1.**



739 **Fig. 2.**



741 **Fig. 3.**



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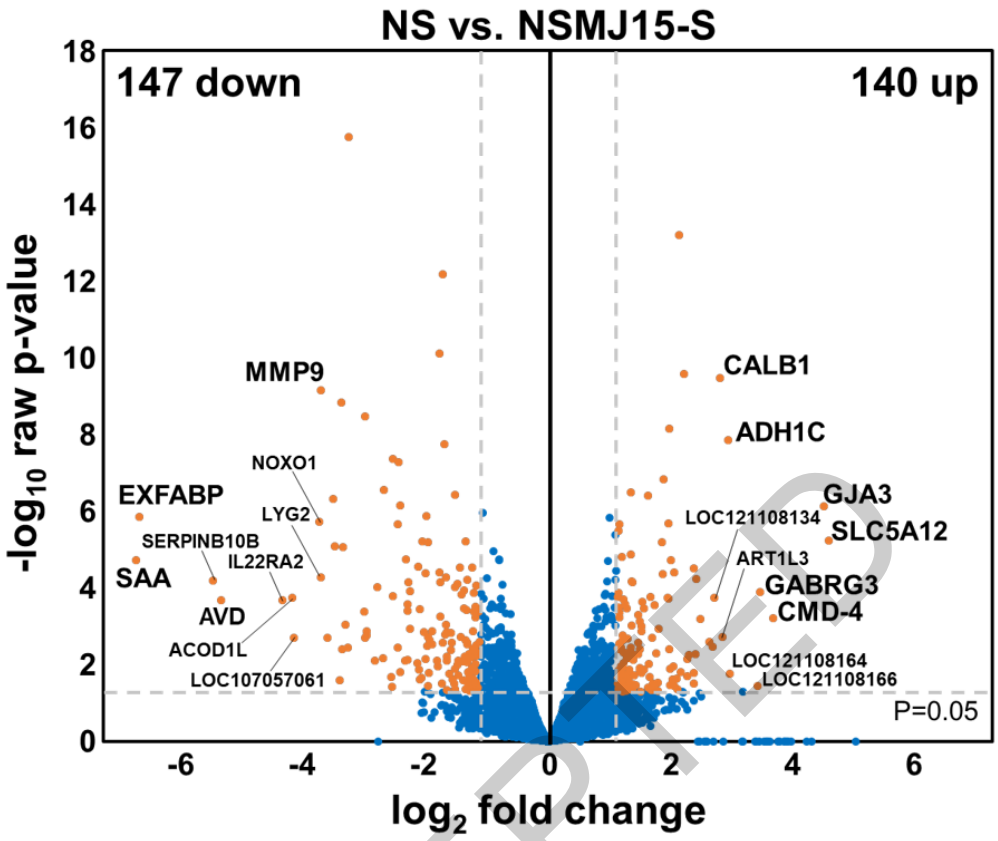
744 **Fig. 4.**



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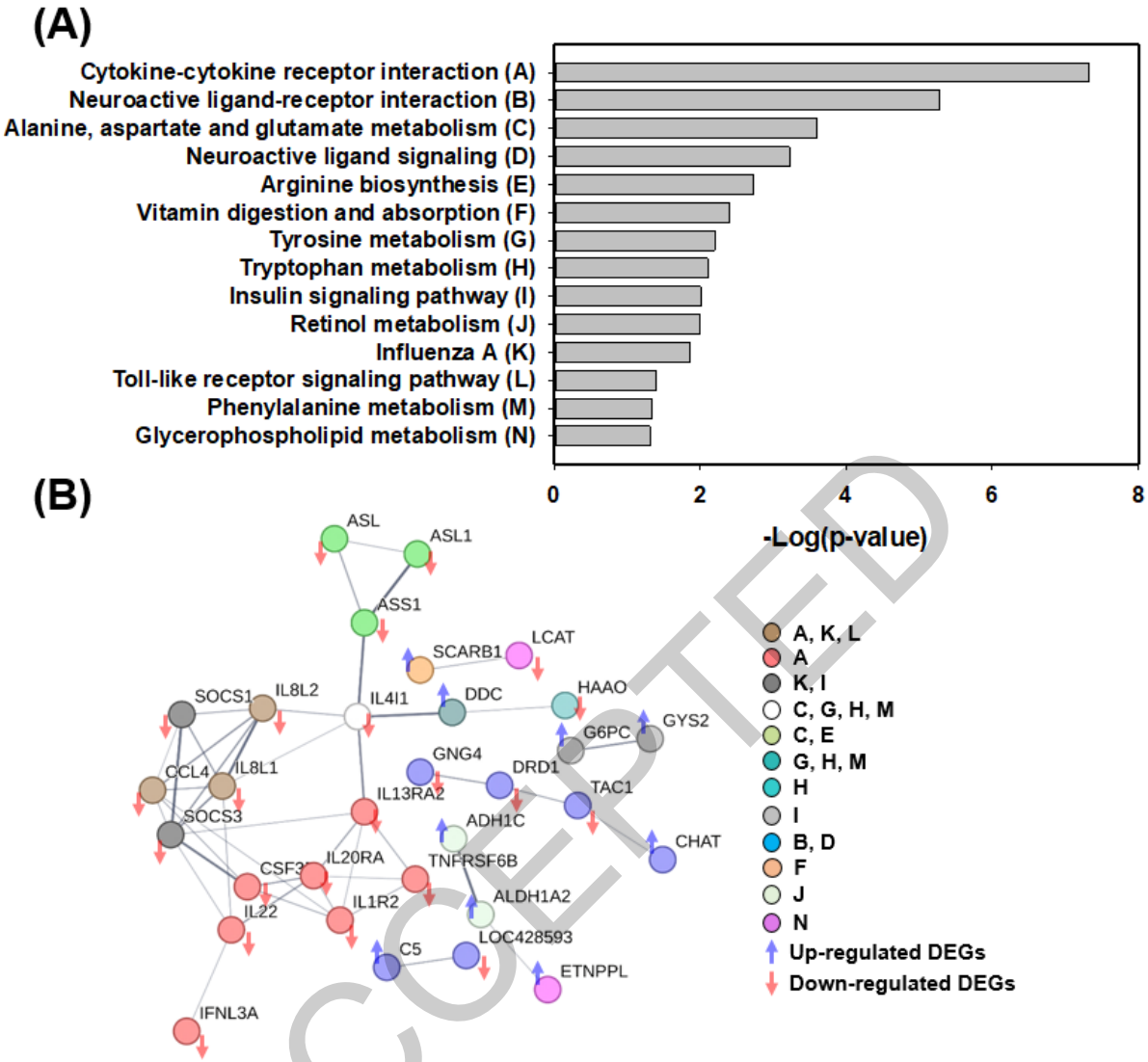
747 **Fig. 5.**



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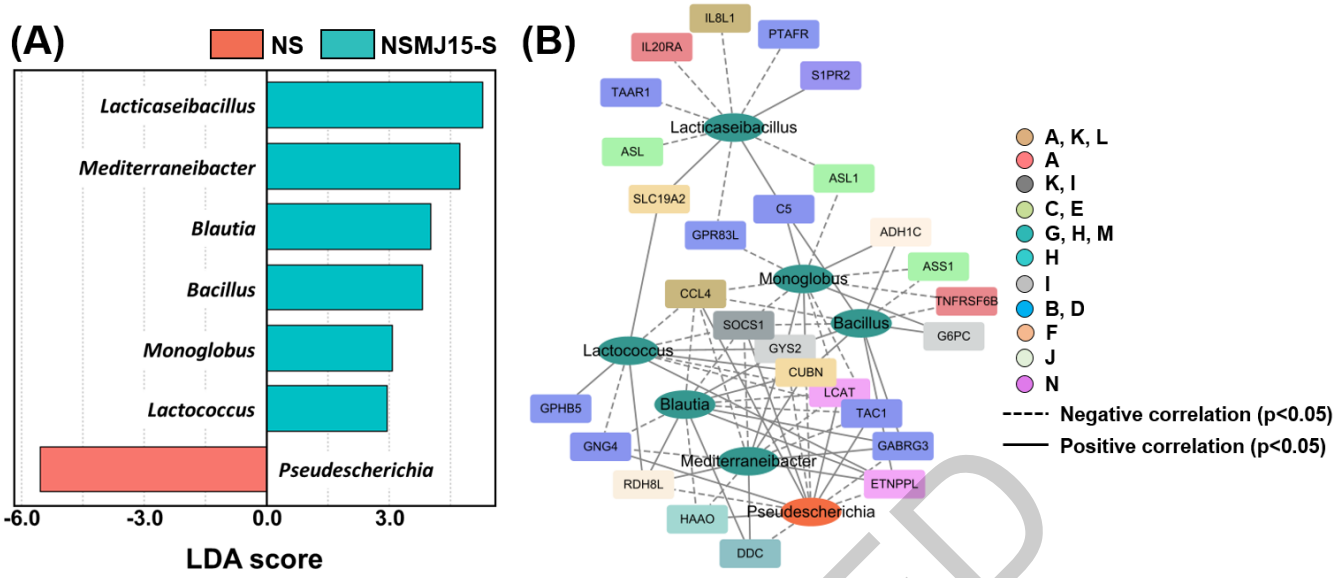
750 **Fig. 6.**



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Supplementary Materials:

Table S1. Ingredients and chemical composition of the experimental diets (as-fed basis)

Item	Experiment diets	
Ingredient composition, g/kg	Control diet	NSMJ15 diet
Corn	550.40	550.40
Soybean meal	360.00	360.00
Ground corn	5.00	–
Lacticaseibacillus paracasei NSMJ15	–	5.00
Soybean oil	20.00	20.00
L-arginine	2.24	2.24
L-histidine	0.52	0.52
L-isoleucine	1.33	1.33
L-lysine-HCl (78.4%)	4.30	4.30
L-methionine	2.81	2.81
L-cystine	1.90	1.90
L-threonine	2.29	2.29
L-valine	1.91	1.91
Limestone	12.61	12.61
Monocalcium phosphate	18.19	18.19
Salt	4.50	4.50
Vitamin premix[1]	5.00	5.00
Mineral premix[2]	5.00	5.00
Choline chloride	2.00	2.00
Calculated composition, g/kg		
Apparent metabolizable energy, kcal/kg	2974.8	2957.1
Crude protein	233.1	233.1
Calcium	9.60	9.60
Non-phytate phosphorus	4.80	4.80
SID amino acids, g/kg		
Arginine	13.69	13.69
Histidine	4.73	4.73
Isoleucine	8.59	8.59
Leucine	14.55	14.55
Lysine	12.79	12.79
Methionine	5.10	5.10
Cysteine	4.40	4.40
Phenylalanine	8.67	8.67
Threonine	8.59	8.59
Tryptophan	2.14	2.14
Valine	9.59	9.59

[1]Vitamin premix was provided per kilogram of diet as follows: retinyl acetate, 24,000 IU; cholecalciferol, 8,000 IU; DL-α-tocopherol acetate, 160 mg/kg; menadione nicotinamide bisulfite, 8 mg/kg; thiamine mononitrate, 8 mg/kg; riboflavin, 20 mg/kg; pyridoxine hydrochloride, 12 mg/kg; D-calcium pantothenate, 40 mg/kg; folic acid, 4 mg/kg; nicotinamide, 12 mg/kg.

[2]Mineral premix was added per kilogram of diet, supplying: iron, 120 mg/kg; copper, 320 mg/kg; zinc, 200 mg/kg; manganese, 240 mg/kg; cobalt, 2 mg/kg; selenium, 0.6 mg/kg; iodine, 2.5 mg/kg.

766 **Table S2.** Growth performance parameters of birds offered the diet containing

767 *Lacticaseibacillus paracasei* NSMJ15 during day 0 to 10

Items	Control group	NSMJ15 group	SE	P-value
Initial body weight, g	39.2	39.4	0.08	0.167
Body weight gain, g	165.7	171.1	3.53	0.317
Feed consumption, g	168.3	172.9	3.42	0.367
Feed coversion efficiency	0.985	0.989	0.0151	0.835
Final body weight, g	204.9	210.4	3.47	0.297

768 SE refers to the standard error.

769

770 **Table S3.** Summary of sequencing metrics and the richness and diversity indices of 16S rRNA
771 amplicon sequencing data from jejunum samples (eight non-supplemented control (NS) and
772 eight NSMJ15-supplemented (S) groups)

Sample ID	Total sequencing reads	Processed reads	ASVs	Chao1	Shannon Index (H')	Gini-Simpson Index	Good's Coverage Estimate
NS1	233,524	100,866	105	105	3.10	0.77	0.99
NS2	203,722	86,887	20	20.5	1.26	0.38	0.99
NS3	269,268	121,615	45	45	1.92	0.56	1.00
NS4	220,232	92,237	257	258.5	3.68	0.78	0.99
NS5	189,604	84,983	51	51	2.71	0.76	1.00
NS6	198,428	81,925	57	57	2.08	0.64	0.99
NS7	218,068	97,055	105	106	2.55	0.57	0.99
NS8	228,834	95,532	111	111	3.77	0.81	1.00
NSMJ15-S1	281,480	118,441	114	114	4.47	0.87	1.00
NSMJ15-S2	271,574	119,169	134	134	4.81	0.93	0.99
NSMJ15-S3	268,368	114,128	252	252.13	3.77	0.84	0.99
NSMJ15-S4	262,832	116,553	148	148.33	2.77	0.68	0.99
NSMJ15-S5	167,852	74,582	87	87	3.88	0.86	1.00
NSMJ15-S6	194,966	84,269	133	133	4.15	0.88	0.99
NSMJ15-S7	195,172	88,725	31	31	1.18	0.34	1.00
NSMJ15-S8	283,606	124,448	86	86.33	2.82	0.74	0.99

773

774 **Table S4.** Summary of RNA-seq sequencing statistics and mapping outcomes for jejunum
775 samples (three non-supplemented control (NS) and three NSMJ15-supplemented (S) groups)

Sample ID	Total raw reads	Clean reads	Q20 (%)	Q30 (%)	Mapped reads (%)
NS1	60,738,214	59,397,974 (97.8)	98.61	95.26	96.03
NS6	76,372,650	74,741,100 (97.9)	98.66	95.42	94.3
NS8	80,565,448	78,570,230 (97.5)	98.46	94.85	96.55
NSMJ15-S1	76,594,660	74,894,908 (94.1)	98.41	94.72	96.17
NSMJ15-S2	61,310,228	60,135,856 (98.1)	98.72	95.57	97.00
NSMJ15-S3	78,488,622	76,521,912 (97.5)	98.38	94.63	96.93

776

777 **Table S5.** Catalog of up- and down-regulated differentially expressed genes (DEGs) identified

778 from jejunal tissue after NSMJ15 supplementation

Fig. S1. Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in the jejunal tissue of ten-day-old broiler chickens following NSMJ15 supplementation. Twelve significantly enriched GO terms (adjusted p-value ≤ 0.05) from the biological process (BP), cellular component (CC), and molecular function (MF) categories are presented as a dot plot. Circle size represents the intersection size, indicating the number of DEGs annotated to each GO term, while the gene ratio denotes the proportion of DEGs assigned to each GO term relative to the total number of DEGs in the corresponding GO category.