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Administration of *L. salivarius* expressing 3D8 scFv as a feed additive improved the growth performance, immune homeostasis, and gut microbiota of chickens

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Abstract

Probiotics have been defined as live microorganisms that are administered in an appropriate amount to provide health benefits to the host animal. In this study, we investigated the effect of *L. salivarius* DJ-sa-01 secreting the 3D8 single-chain variable fragment (3D8 scFv) on the growth performance, cytokine secretion, and intestinal microbial flora of chickens. The experiment was divided into the control group and *L. salivarius* expressing 3D8 scFv experimental group. Chicken was fed 10^9 colony-forming units (CFUs) of wild-type (WT) *L. salivarius* or 3D8 scFv-secreting *L. salivarius* daily for 35 days. The administration of *L. salivarius* expressing 3D8 scFv significantly improved the body weight of chickens compared with the administration of WT *L. salivarius*. A 16S ribosomal RNA metagenomic analysis showed that *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the dominant phyla in both experimental groups. At the genus level, *Lactobacillus* was more abundant (22.82%) in the *L. salivarius*/3D8 group compared with the WT *L. salivarius* group. The serum levels of cytokines, such as IL-8, TNF-α, IL-1β, IFN-γ, IL-4, and IGF1, were significantly reduced in the *L. salivarius*/3D8-treated chickens. In summary, our results suggest that *L. salivarius* expressing 3D8 scFv could be considered a feed additive for improving the growth performance, immune function, and disease resistance of poultry.

KEYWORDS

3D8 scFv, chicken, *Lactobacillus salivarius*, oral administration

1 | INTRODUCTION

Probiotics, which are usually administered in feed, are defined as pure or mixed cultures of live microorganisms that confer beneficial effects on the growth and health of the host by improving the intestinal microbial balance (Brisbin, Gong, Parvizi, & Sharif, 2010). *Lactobacillus* species, which are gram-positive, nonpathogenic microorganisms found in the human and animal intestinal populations (Amit-Romach, Sklan, & Uni, 2004), are one of the most predominantly used probiotics for health benefits. Several studies have revealed that the use of *Lactobacillus*-based probiotics in the livestock industry could result in growth improvements and protection against infection by common enteric pathogens (Gaggìa, Mattarelli, & Biavati, 2010; Mappley et al., 2013). In the last few years, these probiotics have become increasingly popular as nutritional supplements, particularly to reduce gastrointestinal (GI) complaints and common infectious diseases.

3D8 single-chain variable fragment (scFv) is a mini-antibody exhibiting independent nuclease activity against all types of nucleic acids, which is produced from the variable domains of light and

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heavy chains of an anti-DNA antibody that was isolated from the spleen cells of MRL mice (Byun et al., 2017; Kim et al., 2006; Kwon, Lee, Kim, Park, & Shin, 2002). Previous studies have demonstrated that the 3D8 scFv can penetrate into cells via a caveolae-lipid raft pathway (Jang et al., 2009). In a previous study, Hoang et al. (2015) orally administered *Lactobacillus paracasei* (*L. paracasei*) secreting 3D8 scFv to test the antiviral effects of 3D8 scFv against GI viral infections. Cho et al. (2018) reported that *L. paracasei* expressing the 3D8 scFv protein reduces intestinal viscosity and promotes nutrient digestibility in mice. Park et al. (2018) developed an *Escherichia coli* strain harboring a codon-optimized 3D8 scFv gene (*E. coli* 3D8scFv), used this strain as a feed additive against norovirus infection and found that the strain was unable to colonize the GI tracts of mice. Thus, 3D8 scFv could be considered for commercial use as a feed additive in the near future. In fact, the development of an efficient method to deliver therapeutic agents to the mucosal surface has been the main theme of various studies investigating the delivery of probiotics. We hypothesized that 3D8 scFv might exert similar useful effects on the overall health of chicken without inducing any side effects on the normal microbial composition of the intestine. In the present study, we used a chicken model to investigate the impact of *L. salivarius* expressing 3D8 scFv on growth performance, intestinal microbiota balance, and inflammation.

2 | MATERIALS AND METHODS

2.1 | Animal and microorganism

In the present study, 10-week-old rearing hens were used in a 5-week experimental trial. The birds were allocated to two experimental treatment groups and each chicken was orally administered 10^9 colony-forming units (CFUs) of wild-type (WT) *L. salivarius* (WT *L. salivarius*-treated group) or 3D8 scFv-secreting *L. salivarius* (*L. salivarius*/3D8-treated group) daily using a syringe and blunt-end catheter. Each rearing hen was housed in a separate cage (33 × 49 × 60 cm) with free access to water and was fed individually. *L. salivarius* DJ-sa-01 strains were isolated from the chicken small intestine and identified through 16S rRNA gene sequencing (Kim et al., 2018) All chickens were identified using individual tags; water and feed were supplied ad libitum. The experimental protocol used in this study was performed based on an approved animal-use document and according to the guidelines of the animal care and use committee of the National Institute of Animal Science, South Korea (Approval No 2018–273).

2.2 | Construction of recombinant *L. salivarius* expressing 3D8 scFv

The pSLP111.3 expression vector for *Lactobacillus* was provided by Dr. Jos Seegers (Falcobio, the Netherlands). To induce 3D8 scFv protein expression, codon-optimized 3D8 scFv was cloned into pSLP-LDH that was slightly modified from the original vector as previously described (Hoang et al., 2015; Figure 1). WT *L. salivarius* was cultured at 37°C for 2–3 hr before transformation. At the early log phase, *L. salivarius* was harvested and washed twice with phosphate-buffered saline (PBS). 3D8 scFv expression vectors were transformed into *L. salivarius* through electroporation using a Bio-Rad Gene Pulser Xcell electroporator (Bio-Rad Laboratories). After electroporation, transformed *L. salivarius* was incubated anaerobically on MRS plates containing chloramphenicol.

2.3 | Assessment of 3D8 scFv expression in recombinant *L. salivarius*

The expression level of the 3D8 scFv protein in recombinant *L. salivarius* was analyzed using recombinant *L. paracasei* ATCC 334 strains (Hoang et al., 2015) previously seen to express the protein in compliance with the approved protocol of the manufacture (Bioneer Co.). The expression level of 3D8 scFv in transformed *L. salivarius* was analyzed by quantitative real-time PCR using SYBR Premix Ex Taq (TaKaRa) and a Rotor-Gene Q system (Qiagen). The universal 16S rRNA gene, which was used as an internal control, and the 3D8 scFv gene was amplified with the following primers: 16S rRNA (forward 5'-CAYRCCGTAAACGATGARTGCTA-3'; reverse

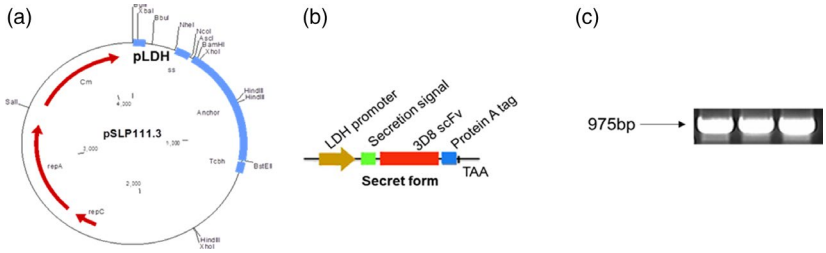

FIGURE 1 Cloning strategy for cell wall secretion of 3D8 scFv via the pSLP-LDH expression vector in *Lactobacillus salivarius*. Codon-optimized and original 3D8 scFv constructs were cloned into the pSLP-LDH vector using the *NcoI* and *AscI* cloning sites (a). Expression cassette designed for secretion of the 3D8 scFv. P_{LDH} *Lactobacillus* dehydrogenase promoter, *Ss* SlpA secretion signal, and *Pro A* protein A tag to prevent fusion of 3D8 scFv (b). The codon-optimized 3D8 scFv gene (975 bp) was cloned into the pSLP-LDH vector for the expression of secreted or cell wall–secreted 3D8 scFv in *L. salivarius* (c)

5'-TAAGGTTCTTCGCGTWGCWTC-3') and 3D8 scFv (forward 5'-GGCAGTATCTGCTGGTGAGA-3'; reverse 5'-CAGTGCCTGAACC ACTACCA-3').

2.4 | Assessment of growth performance and determination of cytokine levels by ELISA

In this experiment, we first checked the body weight gain of the hens every week and recorded the individual body weight gain of each hen. At the end of the experiment, we collected serum samples for determination of the cytokine levels by enzyme-linked immunosorbent assays (ELISAs). The serum concentrations of IL-6, IL-Iβ, IL-8, TNF-α, IFN-γ, IL-4, and IGF1 were determined using commercial chicken ELISA kits (Genorise Scientific, and Wuhan Abebio Science, Co., Ltd.).

2.5 | DNA extraction, pyrosequencing, and data analysis

Total bacterial DNA was extracted from chicken fecal samples using an Extra Master™ Fecal DNA extraction kit according to the manufacturer's recommended protocol (Epicenter). An Illumina 16S rRNA sequencing library was prepared for the V3 and V4 regions, and paired-end sequencing was performed using a 2 × 300-bp paired-end protocol with the MiSeq platform (Illumina) at Macrogen. The sequencing reads of the samples were assigned to a specific sample by their endemic bar codes. The PCR primer sequences, barcodes, and linkers were then removed from the original sequence analysis. For the subsequent analysis, a quality filtering process was used to obtain pyrosequencing reads with more than 300 base pairs and an average quality score of more than 25. A taxonomic alignment of bacterial high-quality sequence reads was performed using the BLAST search tool with the EzTaxon-e database. Sequences that could not be matched to the EzTaxon-e database based on identity species level of 97% were used in a second-order UCHIME program to identify unmatched sequences. Operational taxonomic units (OTUs) were generated using the CD-HIT program with a similarity level of 97%, in accordance to the protocol designed by Edgar (2010). Microbial community richness and diversity indexes, namely, the Shannon-Weaver diversity index, chao1, and Shannon, and Goods library coverage, were determined using the mothur program.

2.6 | Statistical analysis

The data were analyzed using GraphPad Prism statistical software (GraphPad Software). An unpaired *t* test was used for comparisons of the treatment groups, and a P-value less than 0.05 was considered statistically significant. The data are presented as the means ± standard errors (*SE*s) or standard deviations (*SD*s).

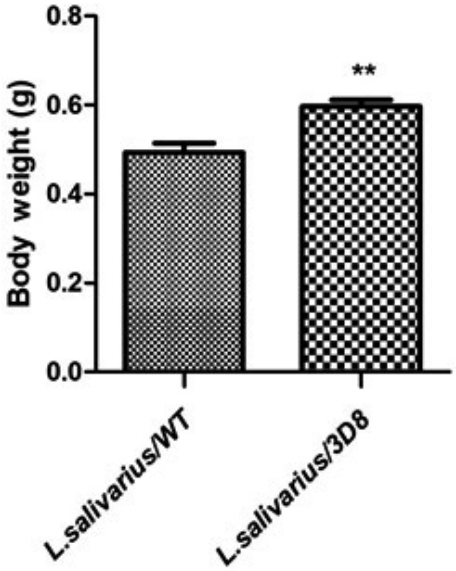

FIGURE 2 Effect of *L. salivarius* secreting 3D8 scFv on growth performance traits in chickens. Statistical comparisons were performed between chickens that were administered WT *L. salivarius* once daily for 35 days and chickens that were administered 3D8 scFv-expressing *L. salivarius* once daily for 35 days. The results are expressed as the means ± *SE*s or *SD*s

3 | RESULTS

3.1 | Growth performance and cytokine expression level after administration of *L. salivarius*

In the present study, the oral administration of *L. salivarius* expressing 3D8 scFv increased the body weight gain of chickens during the overall experimental period compared with the administration of WT *L. salivarius* (Figure 2). In addition, to determine whether *L. salivarius*/3D8 stimulated an immune response that contributed to an antiviral effect, we determined the expression levels of several cytokines in serum samples collected from the experimental birds by ELISAs. No significant difference in the expression levels of IL-6 was found among the different treatment groups. In addition, significantly reduced levels of IL-8, TNF-α, and IL-4 were found in the *L. salivarius*/3D8-treated group compared with the WT *L. salivarius*-treated group. However, IL-1β, IFN-γ, and IGF1 expression was not detected in the *L. salivarius*/3D8-treated group (Figure 3).

3.2 | Microbial taxonomic composition of fecal samples of *L. salivarius*-fed hens

Analysis of the microbial taxonomic abundance in samples from the WT *L. salivarius*- and *L. salivarius*/3D8-treated groups revealed molecular bacterial typing profiles representing 248 bacterial species, and 195 of these species were unique. Of the 248 bacterial species, 156 and 92 species were identified from the WT *L. salivarius*- and *L. salivarius*/3D8-treated groups, respectively, which indicated the

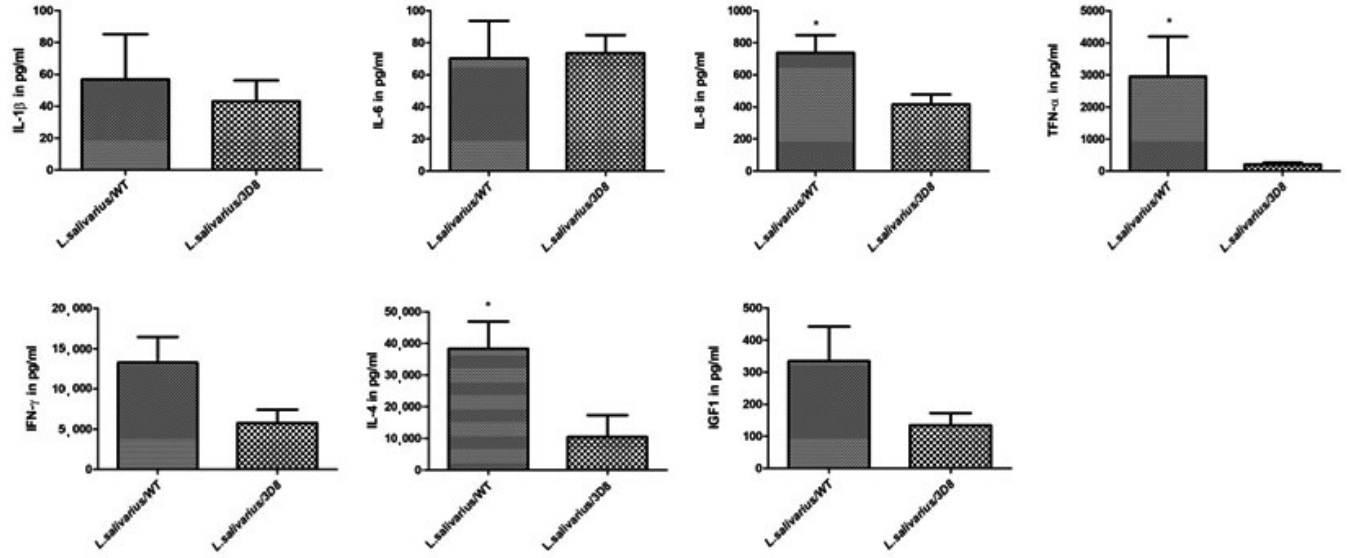

FIGURE 3 Effects of *L. salivarius* secreting 3D8 scFv on serum cytokine levels in chickens. Statistical comparisons were performed between chickens that were administered WT *L. salivarius* once daily for 35 days and chickens that were administered 3D8 scFv-expressing *L. salivarius* once daily for 35 days. The results are expressed as the means ± *SE*s or *SD*s

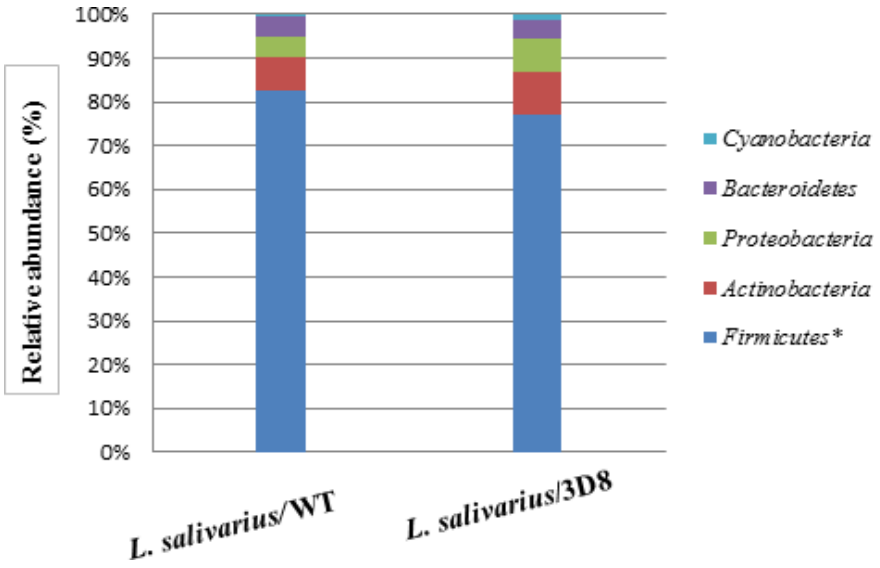

FIGURE 4 Phylum-level bacterial taxonomic composition of the fecal microbiota of chickens that were orally administered *L. salivarius*. Each bar in the stacked bar charts represents the classifications of the total sequences. Asterisks indicate statistically significant differences (*$p > .05$)

existence of different microbial communities in the two groups. The microbial data for the two experimental groups were analyzed to determine the relative abundance percentages (taxon reads/total reads in a sample). As shown in Figure 4, at the phylum level, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the most abundant groups of bacteria in the fecal samples obtained from the *Lactobacillus*-treated groups. The abundance of the phylum *Firmicutes* (82.69%) was significantly increased in the WT *L. salivarius*-treated group. However, the abundances of *Proteobacteria* (9.78%), *Actinobacteria* (7.60%), and *Bacteroidetes* (7.60%) were increased in the *L. salivarius*/3D8-treated group. Interestingly, the unique presence of *Lactobacillus* in the *L. salivarius*-treated groups was expected to provide beneficial effects to the host birds. At the genus level, *Lactobacillus* was highly abundant, representing 22.82% of the composition of the microbiota in the *L. salivarius*/3D8-treated chickens. Other genera found in the microbiota of these chickens included *Blautia* (4.34%), *Ruminococcus* (2.17%), *Clostridium* (1.08), *Enterococcus* (2.17%), *Eubacterium* (2.17%), *Bacteroides* (1.08%), *Corynebacterium (1.08%)*, and *Sphingomonas* (2.17%). The abundances of bacterial species showed significant differences among the tested groups (Figure 5).

4 | DISCUSSION

The poultry industry is facing several challenges, which has increased the public's attention on the potential use of feed additives themselves as well as their potential to deliver beneficial substances

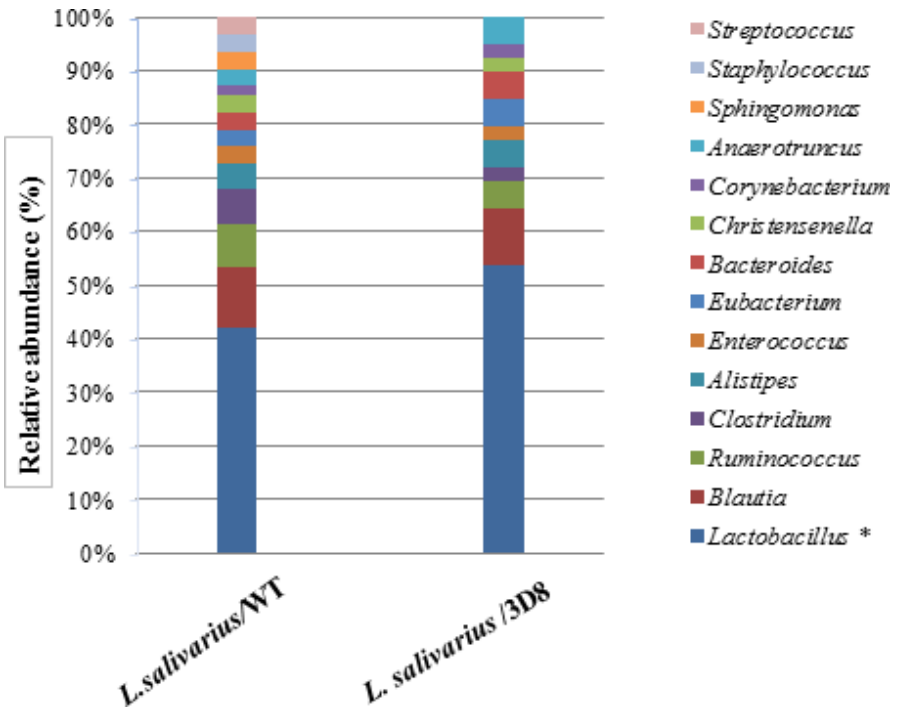

FIGURE 5 Genus-level bacterial taxonomic composition of the fecal microbiota of the chickens orally administered *L. salivarius*. Each bar in the stacked bar charts represents the classifications of the total sequences. Asterisks indicate statistically significant differences (*$p > .05$)

to the GI tracts of the host animals. The data obtained in this study revealed that the oral administration of *L. salivarius* expressing 3D8 scFv increased the body weight gain during the overall experimental period. Previous studies, such as those conducted by Chen, Zhu, and Qiu (2017) and Shokryazdan et al. (2017), revealed that *L. salivarius* administered as feed significantly increases the body weight gain of chickens. Balamuralikrishnan, Lee, and Kim (2017) suggested that *L. salivarius* could increase the body weight gain without affecting the feed intake or feed conversation ratio during the overall experimental period.

Abudabos, Alyemni, Dafalla, and Khan (2017) reported that microbial communities play an important role in the management of host animal health, including feed intake from food, immune system function, and response to GI disease. The administration of *Lactobacillus* provides several health benefits in chickens by improving digestion (Deeth & Tamime, 1981). In the present study, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the most common phyla identified in the fecal samples from the *L. salivarius*/3D8-treated hens, which is consistent with the results reported by Oakley et al. (2013), who found that *Firmicutes* and *Bacteroidetes* are major microbial phyla in chicken feces. Similarly, Cho et al. (2018) reported that *Firmicutes* and *Proteobacteria* provide the greatest contributions to the bacterial taxonomic composition of the fecal microbiota of the *L. salivarius*-treated group at the phylum level. Microbial balance can be stimulated with strains of the genus *Lactobacillus*, which acts as a dominant genus in the small intestine of chickens (Barnes, Mead, Barnuml, & Harry, 1972; Lu et al., 2003). This finding agrees with the results of our genus-level study, which revealed that *Lactobacillus* is the most abundant genus in the *L. salivarius*/3D8-treated group. *L. salivarius* might also control the intestinal environment and/or the growth of intestinal microflora, resulting in the inhibition of diarrhea (Rondon, Ojito, & Arteaga, 2013). Previous studies have shown that the administration of *Lactobacillus* species stimulates the secretion of mucus and promotes the growth of intestinal microflora (Estienne, Hartsock, & Harper, 2005; Yu, Wang, Li, & Qiao, 2008). Effective clarification has been proposed to restore and expand the microbial balance in the intestine and improve the growth promotion of the host.

Interleukins are cytokines that constitute an important component of the immune system. According to Tayal and Kalra (2008), cytokines have an important function, and imbalances in cytokine production result in pathological disorders. In the present study, we found that the IL-8, TNF-α, and IL-4 levels were significantly reduced in the *L. salivarius*/3D8-treated group compared with the WT *L. salivarius*-treated group. These results indicate that the administration of *L. salivarius* expressing 3D8 is interrelated with immunocompetent cells and adjusts the production of proinflammatory cytokines. In addition, the levels of the cytokines IL-1β, IFN-γ, and IGF1 were substantially decreased in the *L. salivarius*/3D8-treated group. Huang and Lee (2018) revealed that *Lactobacillus* supplementation regulates the expression of proinflammatory mediators in the nuclear factor kappa B (NF_kB) signaling pathways, inflammation, and the immune response in chickens, additionally Wimonrat, Jennifer, James, and Somying (2016) agreed with that statement. Brisbin et al. (2010) reported that *Lactobacillus* species reduce antigen-specific IFN-γ production by chicken spleen mononuclear cells. Although the fundamental mechanism for this phenomenon is unknown, it can be speculated that the decrease in IFN-γ production by splenocytes of chicken fed *Lactobacillus* species might reflect a selective decrease in Th1 cell activation. Cytokines are critical for chicken health, and hence, any alterations in cytokine production cause pathological disorders (Tayal & Kalra, 2008). Th1 cells primarily produce IFN-γ,

TNF-α, IL-6, and IL-2, which are considered proinflammatory cytokines. However, IL-4 and IL-8 are anti-inflammatory cytokines secreted by Th2 cells (Romagnani, 1995). Zhang et al. (2011) suggested that the dietary inclusion of *L. salivarius* increases the expression of the IL-6 gene in the chicken intestine. However, in the present study, we did not find a significant difference in IL-6 expression between the treatment groups. Lamprecht et al. (2012) previously reported that IL-6 and TNF-α are associated with independent pathways and that the proinflammatory cytokine IL-6, which is a central mediator of the systemic inflammatory response, is not influenced by *Lactobacillus* supplementation. These results suggest that the administration of *L. salivarius* expressing 3D8 prevents activation of the immune system and maintains immune homeostasis. Together, these results indicate that the observed improvement in the body weight gain in chickens is due to the increased digestion efficiency and improved immune homeostasis obtained after the administration of *L. salivarius* expressing 3D8 scFv.

5 | CONCLUSION

In summary, our results revealed that the administration of *L. salivarius* expressing 3D8 scFv can be considered a feed additive that could be used to improve the growth performance and reduce the inflammatory responses without affecting the microbial composition in the chicken intestinal microbiota. Therefore, in the future, the expression of 3D8 scFv in recombinant *L. salivarius* might constitute a good alternative for improving growth performance in the livestock industry.

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