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Original Article

Preliminary Study to Investigate the Effect of Lactobacillus Reuteri Administration on Growth Performance, Immunological, Gut Microbiome and Intestinal Mucosa of Chicken

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Probiotics, growth performance, gut microbiome, broiler.

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ABSTRACT

The present study was conducted to investigate the efficacy of oral administration of *L. reuteri* on growth performance, intestine histomorphology, immunological and gut microbiome of broilers. A total of twenty healthy chickens were used in a five-week experimental trial. Birds were assigned into one of two groups with orally administrated *L. reuteri* probiotic and without probiotic- (Control -Phosphate-buffered saline). A significant ($p<0.05$) body weight gain was observed in the chickens in *L. reuteri* treatment group compare to those in the control group at the end of the trial. In addition, the serum IGF-1 cytokines level significantly enhanced in *L. reuteri* treatment group. However, there were no notable effects observed on the villus height, crypt depth, muscularis thickness, and submucosal thickness in chickens orally inject with and without *L. reuteri*. At the phylum level, the presence of *Firmicutes* (99.5%) was highly abundantin the *L. reuteri* treatment group. Moreover, the fecal microbial communities of *Lactobacillus* (99.9%) showed average relative abundance at genus level in *L. reuteri* treatment group. From this, we concluded that oral administration of *L. reuteri* would be beneficial to enhance the body weight gain, gut microbiome, and immune status of broiler.

INTRODUCTION

Broilers are widely used as an equitable meat source of protein globally. Compared to other meat-producing animals, these broilers grow faster and meet the customers' protein needs in the shortest period (Castonon, 2007). Such, broiler meat and eggs have become the cheapest animal protein source for human consumption and plays a significant role in enhancing the health status of humans (Rudra *et al.*, 2018).However, rearing broiler is not an easy task as they are caged in confined spaces, and may have the potential for rapid disease spread among poultry flocks (Bhogoju *et al.*, 2021) thereby affecting the productivity and cause a huge economic loss for poultry producers. In earlier days, antibiotics were widely used in poultry feedstuff as a growth promoter (Ogle, 2013), however, the overuse of certain antibiotic leads to bacterial resistance in animals and negatively affects the consumers health through food chain (Upadhaya *et al.*, 2016). This circumstance has prompted poultry producers and nutritionist to find alternative solution to AGP, however finding delicate feed additive that could improve the animal health and productivity become a challenging mission. After a long quest, prebiotic, probiotic, organic acid, emulsifiers, phytogenic, etc., were found to be the best alternatives. Among those applications, probiotics have been considered as one of the best alternatives and is used the poultry diet since many decades with a promising result (Smith, 2014).

A nutritional approach on the use of probiotics has gained more attention since the 1970s (Fuller, 2012) and it's been widely addressed by many researchers that probiotic, live microorganisms used as therapeutic adjuvants could improve the feeding behavior and reduce morbidity and mortality of animals (Abdel-Azeem, 2013). Recently, probiotics were used in different strains with different efficacies which include *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus reuteri (L. reuteri)*, *Lactobacillus salivarius*, *Bifidobacterium bifidum*, *Bifidobacterium longum, Enterococcus faecalis, Enterococcus fecium, Streptococcus cremoris, and Streptococcus salivarius* were proven to provide certain benefits to the host (Patil *et al.*, 2015; Bhogoju *et al.*, 2021). Amongst, lactic and acetic acid bacteria were widely used to produce antimicrobial substance against the homologous strain and produce microbicidal substances against gastric and intestinal pathogens (Ljungh & Wadstrom, 2006). Apart from this, *Lactobacillus* strains are very effective in diminishing *Escherichia coli, Salmonella*, and *coliform* counts in poultry (Hardy *et al.*, 2013). Similarly, Abudabos *et al.* (2013) reported that probiotics had enhanced the animal performance by modifying the intestinal microbiota. Also, Timmerman *et al.* (2004) described that probiotic *Lactobacillus* species increase the growth performance and reduce the mortality in broilers. On the other hand, Suresh Kumar *et al.* (2020) noted that Oral administration of *L. reuteri* expressing 3D8 scFv probiotic improved growth performance, immune function, and gut microbiome in chickens. The existence a microbial population is widely diverse within the gastro-intestinal track of an animal (Yeoman & White, 2014) and these bacteria are mainly belonging to phylum bacteria like Bacteroidetes and Firmicutes. Notably, Bacteroidetes comprised with predominant genera like *Bacteroides* and *Prevotella*, while *Firmicutes* phylum consist with *Bacillus*, *Lactobacillus*, *Enterococcus, Clostridium*, and *Ruminicoccus* (Rinninella *et al.*, 2019). The Actinobacteria phylum is comparably less in abundant and mainly characterized by *Bifidobacterium* genus (Arumugam *et al.*, 2011). Bacteroidetes were found in the cecum of chickens, whereas Firmicutes are largely found in the hindgut of pigs. In 2018, Lu *et al.* reported that the inclusion of probiotic complex (*Enterococcus faecium* DSM 7134, *Bacillus subtilis* AS1.836 and *Lactobacillus paracasei* L9) had significantly increased relative abundance of *Prevotella_1* and *Lactobacillius* reduced the relative abundance of *Bacteroidales* and *Clostridium_sensu_1* in weaning pig.

Previously, Wang *et al.* (2014) stated that transformed *L. reuteri* XC1 supplementation had improved the feed efficacy of broiler in a 42 days trial. Though many reports (Wang *et al.*, 2014; Bhogoju *et al.*, 2021) addressed the positive effects of *L. reuteri* on broiler performance, according to our knowledge, effects of *L. reuteri* on intestine histomorphology, immunological and gut microbiome in broiler is still not well elucidated. Therefore, our research team has planned to investigate the efficacy of oral administration of *L. reuteri* supplementation on the growth performance, serum immunological response, fecal microbiota, and intestine histomorphology in broiler chickens.

MATERIALS AND METHODS

This experiment was conducted at the National Institute of Animal Science (NIAS) "Poultry farming unit" (Jeonju, Jeollabuk-do, Republic of Korea) in strict accordance with the guidelines of the Institutional Animal Care and Use Committee. Prior to the trial, the research protocols were well revised and approved (2018-273) by the Ethics Committee of NIAS, Jeonju, Republic of Korea.

Before starting the trial, all equipment and rearing houses were disinfected. A total of 20 chickens (10 wk- old) were weighed, separated into two groups (10/treatment and 1/cage): CON and *L. reuteri*, and individually distributed in multi-layer battery cages. For a period of 5 weeks, CON group chicks were orally injected with Phosphate-buffered saline (PBS) while, treatment group chicks were orally injected with *L. reuteri* (10^9colony-forming units (CFUs) wild-type strains. The probiotic strain (*L. reuteri* SKKU-OGDONS-01*)* employed in this study was prepared according to the method described by Kim *et al.* (2018). First, the room temperature was maintained at 30 ± 1°C and gradually reduced up to 24°C (60% humidity) and maintained throughout the trial. Each cage was equipped with a nipple drinker and a feeder, allowing the birds to *ad libitum* access feed and water during the whole experiment period. To maintain a hygienic environment rearing house, it was cleaned every week until the end of the study.

The body weight of an individual bird was measured every week to determine the average daily gain (ADG). On day 35, blood samples (5 ml) were collected from the brachial veins of 20 birds using a sterile syringe and kept in (K3EDTA) (Becton, Dickinson, and Co., Franklin Lakes, NJ, USA) heparinized and non-heparinized

tubes. Within one hour of collection, all samples were centrifuged (3,000×g) at 4 °C for 15 min to separate the serum. Enzymatic kits (Roche Diagnostics GmbH, Mannheim, Germany) were used to determine the levels of pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-4, IL-6, IL-8, IL-1β, Interferon gamma (IFNγ), and Insulin-like Growth Factor-I (IGF1) in the bird's serum were determined by using chicken ELISA kits (Genorise scientific, Co., Ltd., China) according to the manufacturer's instructions.

Duodenum and jejunum mucosa morphology were analyzed by the methods of Balasubramanian *et al.*, (2021). In brief, the intestinal segments were fixed in 4% paraformaldehyde, then embedded in paraffin, and stained with hematoxylineosin. Villus height and crypt depth were measured in 40 × magnification with a digital camera microscope (BA400Digitl, McAudi Industrial, 7 / 32 Group Co., Ltd). A total of 10 intact villi and crypts were randomly selected in each sample. Then, the data included villus height, crypt depth and their ratio (V/C) was calculated.

On day 35, 200 g (each treatment) of fresh excreta specimens were collected from 20 birds placed in the icebox, and taken to the laboratory. Metagenomic DNA (mDNA) was extracted according to manufacturer instructions. 100 mg of excreta sample was added into 1.4 ml lysis buffer (2 ml tube) and samples were thoroughly homogenized using Gilson vortex mixer. Subsequently samples were mixed with 0.2 g sterile zirconia/silica beads. Then, the samples were processed on a Tissue Lyser at 30 Hz for about 6 min. Lysis was completed within 5 min at 95 °C. Finally, DNA was extracted following the instructions for the QIAamp Power Fecal Kit (Qiagen, Germany) and eluted in 100 µl elution buffer provided in the kit. The following protocol describes the steps carried out to amplify the targeted 16S rRNA gene V3-V4 regions of the bacteria present in each of the collected samples, as well as processes required to prepare the purified DNA fragments for next-generation sequencing. The preparation of a library of amplicons consisting of 16S rRNA gene and sequencing was done by the Illumina MiSeq platform. Operational taxonomic units (OTU) were clustered with a similarity of 97% using UPARSE software. Gene sequencing and analysis data were performed at Macrogen Co. Ltd. (Seoul, Republic of Korea).

Statistical Analysis

Data were expressed as means ± SE. Statistical differences among groups were tested by one-way analysis of variance (ANOVA). An unpaired t-test was used for the treatment group comparison. GraphPad Prism 9.0 was used for statistical analyses. Values were considered significantly different when $p<0.05$.

RESULTS AND DISCUSSION

Since the phase-out of AGP in poultry feed, probiotics have been studied for their potential to improve growth performance in commercial chicken production. The efficacy of oral administration of *L. reuteri* supplementation on growth performance on broilers are shown in Fig.1. Compared to CON, the broilers that received *L. reuteri*/WT significantly enhanced the body weight gain at the end of the trial; this was constant to Liu *et al.* (2005) and Sureshkumar *et al.* (2021) who found a similar improvement in the daily gain of broilers fed dietary supplement with *L. reuteri.* Additionally, Sattler *et al.* (2014) reported that probiotic strain *L. reuteri* had a beneficial effect on chicken growth thereby improving intestinal health. Previously, Awad *et al.* (2010) reported that the administration of *L. salivarius* and *L. reuteri* positively influence the body weight of broiler chicks at the finisher stage. One of the possible reasons for better growth of broilers in this study might be due to the healthy intestine morphology.

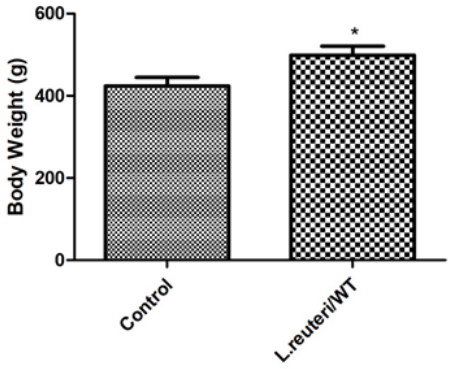

Figure 1 – Body weights from the chickens after administration of *L. reuteri*/WT. Statistical comparisons were made between the control group and the *L. reuteri* group. Values were considered significantly different when $p<0.05$.

The main function of probiotics is to promote the immune status of animals by preventing the invasion of harmful intestinal infections (Panda *et al.*, 2007). As shown in Fig 2. ELISA was used to measure the serum concentrations of TNF-α, IL-4, IL-6, IL-8, IL-1β, IFNγ, IGF1 in two groups. The level of IGF-1 was significantly enhanced in L. *reuteri*/WT treatments group compared to CON group. However, there was no difference observed on TNF-α, IL-4, IL-6, IL-8, IL-1β, and IFNγ cytokines level of birds. Interleukins are cytokines that constitute an important component of immune system. Previously, Kim & Lillehoj (2019) demonstrated that

probiotic have the potential to balance proinflammatory cytokines by boosting the anti-inflammatory cytokines like IL-10 and TGF-β. Excessive production of pro-inflammatory cytokines like IL-12 and IFN- are inflamed in bowel and has been shown to have a devastating effect on the intestinal tissue destruction (Pallone & Monteleone. 2001). To date, administrating broilers with *L. reuteri*, results in obtaining a high effect on the serum cytokines concentration, althoughthere is not sufficient comparison for further elucidation.

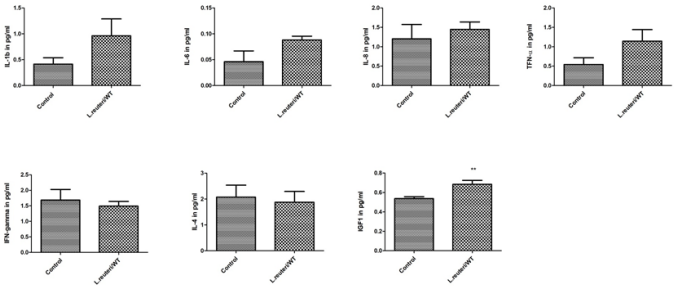

Figure 2 – Effects of *L. reuteri* serum cytokine concentrations in chickens. Statistical comparisons were made between chickens that received control and *L. reuteri*/WT oral administration for 35 days. Values were considered significantly different when $p<0.05$.

The immune organs of birds can be characterized into peripheral and central immune organs (Song *et al.*, 2021). The central immune system can participate in the immune response by culturing mature functional lymphocytes without antigenic stimulation and then exporting these lymphocytes to the peripheral immune system. The higher proliferative activity of peripheral blood lymphocytes is indicated by the functional activity of T and B cells (Dekruyff *et al.*, 1975). Such T and B lymphocytes becomes acritical component of the immune system's and highly reacts to stress and/or hostility (Naukkarinen *et al.*, 1989).Also, the level of T and B cells may serve as a fungible indicator of lymphocyte immunity. Besides, the immunological balance was mainly related to changes in Th1 and Th2 cytokines, and *L. reutri*s trainscould help to maintain the immune homeostasis. According to Xu *et al.* (2003),the small intestine is the primary site of nutrient absorption, and the rate of nutrient absorption is determined by intestinal morphology, structure, and function. The data for the morphological measurements of the chicken's intestines are presented in Fig. 3. There were no notable effects observed on the villus height, muscularis thickness, and submucosal thickness in chickens fed with and without *L. reuteri* supplemental groups. However, the dietary administration of *L. reuteri*/WT treatment group had markedly increased crypt length in the jejunum compared to the control treatment group. Previously, Al-Sultan *et al.* (2016) stated that probiotics supplementation had improved the villus height and reduced the crypt depth in the small intestine of broilers. Similarly, Alagawany *et al.* (2007) stated that a multi-microbe probiotic containing *L. acidophilus*, *L. casei*, and *Enterococcus faecium* had increased the jejunal villus length and decreased the villus crypt depth. The intestinal epithelium permits to absorb the nutrients by preventing the invasion of infections entering into bloodstream. In 2020, Meyer *et al.*, discovered that adding *Lactobacillus plantarum* and *L. reuteri* to broiler feed improved barrier integrity and blocked the entering of pathogenic microorganisms. Moreover, Song *et al.*, (2014) found that a blend of probiotic (*B. licheniformis*, *B. subtilis,* and *L. plantarum*) had helped broilers to reduce the heat stress-induced by gut microbiota, barrier integrity, and histomorphology.

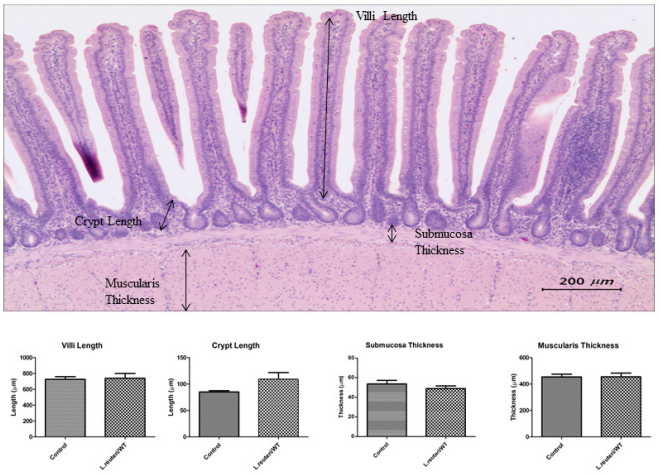

Figure 3 – Morphometric changes in the intestinal mucosa of the jejunum after *L. reuteri* administration in chickens. Villi length, crypt length, muscularis thickness and submucosal thickness are indicated with black arrows. Values were considered significantly different when $p<0.05$.

The balance between host immune system and gut microbiome plays a crucial role in health and disease. Although pathogen-induced microbiota disturbances have been associated with a variety of intestinal and systemic diseases, beneficial bacterial colonization is often associated with high broiler output (Danzeisen *et al.*, 2013; Wideman, 2016; Clavijo & Florez. 2018). In this study, compared to the CON group *L. reuteri*/WT group broilers showed a total of 177,088 bacterial abundance. Firmicutes and Bacteroidetes are the most dominant phyla in piglets' fecal sample (Lu *et al.*, 2003). The comparative abundances of the excreta microbiota phyla between the control and *L. reuteri* groups is shown in Fig 4. Almost 99.5% *Firmicutes* were dominant phyla in the *L. reuteri*/WT group. However, *Bacteroidetes, Proteobacteria, Cyanobacteria,* and *Actinobacteria* phyla bacterial abundant were also present in this group. At the phylum level, 95% relative

abundance of *Firmicutes* and 3% of *Cyanobacteria* were highly abundant in control group, respectively. The average relative abundance of *Proteobacteria, Actinobacteria,* and *Bacteroidetes* were found in control excreta samples. This is also in agreement with Cho *et al.* (2018) who found an *Firmicutes* and*Proteobacteria* microbial communities dominantly presented in *L. reuteri* treatment group at the phylum level. Considering that the adequate balance between *Proteobacteria,* and *Firmicutes* phyla is an evaluation point for intestinal bacterial composition in healthy animals, we could confirm that probiotics positively affected the broilers intestinal microbial environment.

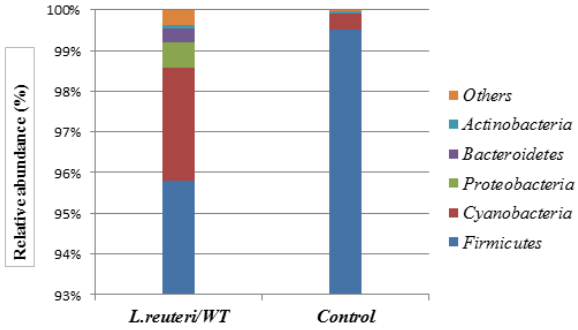

Figure 4 – The microbial communities with an average relative abundance of fecal microbiome at phylum level in between the control and *L. reuteri*/ WT groups. Each bar in the stacked bar charts represents the classifications of the total sequences.

The microbial communities with an average relative abundance of fecal microbiome at genus level is shown in Fig 5. At the genus level, *Lactobacillus* microbial community was dominantly abundant in the *L. reuteri*/ WT group (99.9%) compared to control group. Similarly, Barnes *et al.* (1972) and Lu *et al.* (2003) stated that chickens fed with probiotic supplementation have a dominant *Lactobacillus* genus. On the other hand, Sonnenburg & Backhed (2016) stated that *Lactobacillus* spp. can quickly create a complex bacterial community and protect the host from harmful bacteria infections. In addition, the intestinal microbiota affects the physiological development, health, and productivity, leading to the hypothesis that the use of feed additives such as organic acids can be useful to control microbial community (Upadhaya *et al.*, 2021). Previously, Kim *et al.* (2014) reported that *Prevotella* and *Arcobacter* are important acetate-producing bacteria. The effective bacteria might be proposed to restore and expand the microbial balance in the intestine thereby improving the growth of chickens. *Lactobacillus* is one of the most common bacterial genera in broiler chicken intestines, and it has both direct and indirect health benefits (Wei *et al.*, 2013). We assume that the administration of *L. reuteri* to broilers has highly help them to maintain an intestinal bacterial environment and that could positively enhance their body weight.

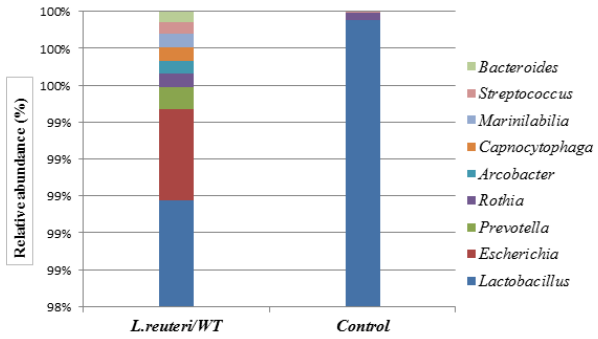

Figure 5 – The microbial communities with an average relative abundance of fecal microbiome at genus level in between the control and *L. reuteri*/ WT groups. Each bar in the stacked bar charts represents the classifications of the total sequences.

CONCLUSION

Our result demonstrated that oral administration of *L. reuteri* would be beneficial to increase the growth and immune status of broilers by improving their gut health. However, there was no difference observed on the intestine histomorphology of the villus height, muscularis thickness, and submucosal thickness in chickens administrated with and without *L. reuteri.* As this is a preliminary study, we are not able to explain the exact reason for this outcome, at the same time our research team has planned to conduct further studies in-depth.

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CONFLICT OF INTEREST

All authors have declared that they have no potential conflict of interest.

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