

Effects of Probiotic *Lacticaseibacillus paracasei* NSMJ27 on Laying Performance and Gut Health Indicators in Aged Laying Hens

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Simple Summary

Probiotics are live beneficial microbes that are known to improve the performance, health and welfare of laying hens. In our attempts to develop novel probiotic strains for poultry, *Lacticaseibacillus paracasei* NSMJ27, isolated from Korean fermented foods, gained our attention for its probiotic potential. It is known that dietary probiotic strains, although they exert beneficial potentials in vitro, require in vivo validation with chickens to verify biological effectiveness. In the present research, we attempted to evaluate the effect of a novel probiotic strain on gut health in aged laying hens. Our study shows that *L. paracasei* NSMJ27 enhanced antioxidant capacity and improved gut health, suggesting its potential use as a feed additive for laying hens.

Abstract

This experiment was designed to determine the effect of the *Lacticaseibacillus paracasei* (*paracasei*) strain NSMJ27, isolated from Korean fermented vegetable food (home-made kimchi), on laying performance, egg quality, intestinal histology, cecal short-chain fatty acids, and ileal antioxidant/immunity indicators of laying hens. Ninety-six 55-week-old Hy-Line Brown hens were randomly assigned to two dietary treatments with each treatment comprising eight replicates of six hens each. Experimental diets were prepared by mixing corn and soybean meal basal diets without or with *L. paracasei* NSMJ27 at 2.5×10^9 CFU/kg. The experiment lasted 4 weeks. Laying hens fed with the NSMJ27-supplemented diet were not affected ($p > 0.05$) in their laying performance or egg quality. With respect to ileal morphology, villus height: crypt depth ratio tended to be higher ($p = 0.067$) in laying hens fed with *L. paracasei* NSMJ27 vs. control diets. Dietary *L. paracasei* did not affect ($p > 0.05$) the activities of glutathione peroxidase and catalase, malondialdehyde contents, or secretory immunoglobulin A in ileal mucosa, but increased ($p = 0.048$) the activity of superoxide dismutase compared with the control diet-fed laying hens. Dietary *L. paracasei* elevated



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($p = 0.016$) the relative percentage of butyrate but lowered ($p = 0.057$) that of isovalerate in cecal digesta. Dietary *L. paracasei* did not affect the percentages of cells expressing macrophages, B cells, CD4⁺ T cells, CD8⁺ T cells, or TCR $\gamma\delta$ T cell surface markers ($p > 0.05$). Overall, these results suggest that dietary *L. paracasei* NSMJ27 could enhance gut health via increasing gut antioxidant capacity and butyrate production in the cecal digesta of laying hens.

Keywords: laying hen; *Lactobacillus paracasei*; laying performance; egg quality; gut health; short-chain fatty acids; antioxidant; immunity

1. Introduction

The avian gastrointestinal tract fulfills dual critical functions, serving as both a primary digestive organ and the largest immune barrier within a bird's body [1], and harbors a well-balanced gut microbiota crucial for maintaining optimal gut health [2]. In aged laying hens, physiological aging leads to gut dysbiosis, impaired intestinal barrier function, weakened immunity, and reductions in egg production and quality due to oxidative stress and microbiota shifts [3–6]. To maintain a balanced intestinal ecosystem in chickens, dietary probiotics are frequently supplemented [7], providing essential health benefits through the administration of adequate viable microbes [8]. In addition, probiotic inclusion in layer diets has been known to improve egg quality, feed efficiency, nutrient utilization, and overall flock health with less dependence on in-feed antibiotics [9–12].

Lactobacillus species are known to possess antibacterial properties against various enteric pathogens and potentially improve gut microbiota composition [10]. Among them, *Lactobacillus paracasei* (*L. paracasei*) is a Gram-positive, lactic-acid bacterium naturally found in fermented dairy products and the animal intestinal tract [11]. *L. paracasei* has been shown to possess several probiotic properties that could be beneficial for animal health. These properties include the suppression of mucosal inflammatory cytokines such as tumor necrosis factor alpha and interleukin 12, the mitigation of intestinal inflammation, the enhancement of secretory immunoglobulin A (sIgA) levels in the small intestine, and the modulation of gut microbiota [12]. It has been reported that dietary *L. paracasei* subsp. *paracasei* L1 enhanced growth performance, feed intake, and cecal microbiota diversity in chickens [13], while unclassified *L. paracasei* improved feed conversion ratio, body weight gain, and antioxidant status in *E. coli*-challenged broilers [14]. The beneficial health or growth-promoting effects of *L. paracasei* strains were also reported in murine models [15] and pigs [16].

L. paracasei strain NSMJ27, recently isolated from Korean fermented foods, appears to be a particularly promising candidate due to its tolerance to the harsh gut environment (e.g., at low pH values and high bile salt concentrations) characteristics commonly found in the gut [17]. In our ongoing attempts to find a novel probiotic candidate for chickens, we reported the beneficial effect of the *L. paracasei* NSMJ56 strain, which enhanced egg quality, exhibited antioxidants, and modulated cecal microbiota composition in young laying hens [18]. Although *L. paracasei* species has been used in poultry, no studies have been tested with recently isolated *L. paracasei* NSMJ27 strain on cecal short-chain fatty acid (SCFA) profiles, ileal antioxidant status, or immune responses in aged laying hens. Therefore, the purpose of this communication was to elucidate the potential benefits of probiotic *L. paracasei* strain NSMJ27 supplementation with emphasis on cecal short-chain fatty acids and ileal antioxidant/immune status in aged laying hens.

2. Materials and Methods

2.1. Ethics Approval Statement

All experiment protocols received approval from the Institutional Animal Care and Use Committee in Konkuk university (KU 21083), Republic of Korea.

2.2. Animals Experimental Design

Initially, 12-week-old Hy-Line Brown pullets were obtained from a local commercial farm (Eunha farm, Cheonan-si, Chungcheongnam-do, South Korea) and reared at a KU research farm (Chungju-si, Chungcheongbuk-do, South Korea). In this study, laying hens that had been raised at the KU research farm were selected at 53 weeks of age from a flock of 300 hens to have similar laying performance. Before the initiation of the experiment, egg weights and egg production were monitored for 2 weeks to ensure that all hens were producing and treatment groups had equal laying performance. Ninety-six 55-week-old Hy-Line Brown laying hens were randomly allocated to two dietary treatments (eight replicates of 6 hens each) in the 3-tier multi-layer battery, evenly distributed across all tiers and rows with an alternating arrangement. A mash-type diet based on corn–soybean meal was prepared (Table 1). Experimental diets were prepared by mixing a basal diet supplemented without or with lyophilized *L. paracasei* NSMJ27 at the content of 2.5×10^9 CFU/kg. This inclusion level was determined based on a prior trial with *L. paracasei* NSMJ56 strain [18], which reported to influence immunity and microbiota in 21-week-old laying hens fed on a diet containing 5×10^8 CFU/kg of NSMJ56 strain. In addition, aged layers typically exhibit compromised gut health requiring higher probiotic doses to achieve improvements in gut function and subsequently enhance laying performance. According to Alaqil et al. [19] and Wang et al. [20], dietary supplementation at 2×10^9 or 3×10^9 CFU/kg probiotics showed positive effects on the laying performance and egg quality of aged laying hens. *L. paracasei* NSMJ27, isolated from Korean fermented vegetable food (home-made kimchi), was shown to be viable even at low pH or bile salt environment [17]. The *L. paracasei* NSMJ27 strain was prepared in a lyophilized form which had been cultured in MRS medium, centrifuged, resuspending in 10% skim milk, and freeze-dried as described [21]. The basal diet devoid of feed additives was considered the control diet. Each cage (45 cm × 45 cm × 45 cm) housed two hens, and three adjacent cages were considered replicates. This setup aligns with optimized industry housing systems, safely accommodating up to 2 laying hens per cage without welfare compromise. Feed and water were supplied ad libitum for 4 weeks. The chicken room maintained a 24 ± 2 °C ambient temperature and 60% relative humidity, and a 16L:8D light schedule was applied.

Table 1. Ingredients and nutrient composition of the basal diet.

Ingredients	g per 100 g of Diet
Corn	61.65
Soybean meal, 47% crude protein	17.63
Tallow	2.69
Corn gluten meal	3.42
Limestone	11.53
Monocalcium phosphate	1.82
Sodium bicarbonate	0.21
Sodium chloride	0.27
Choline chloride	0.05
L-Lysine HCl-56%	0.20
DL-Methionine-99%	0.13
L-Threonine-99%	0.09
L-Valine-99%	0.11

Table 1. *Cont.*

Ingredients	g per 100 g of Diet
Vitamin premix ¹	0.10
Mineral premix ²	0.10
Calculated chemical composition	
Nitrogen-corrected apparent metabolizable energy, kcal/kg	2700
Dry matter, %	88.53
Crude protein, %	15.0
Ether extract, %	4.89
Ash, %	15.5
Calcium, %	4.40
Available phosphorus, %	0.40
Lysine, %	0.79
Methionine + Cysteine, %	0.64
Threonine, %	0.65
Valine, %	0.81

¹ Vitamin premix provided the following nutrients per kg of diet: vitamin A, 20,000 IU; vitamin D₃, 4600 IU; vitamin E, 40 mg; vitamin K₃, 4 mg; thiamin, 3.6 mg; riboflavin, 8 mg; pyridoxine, 5.798 mg; cobalamin, 0.04 mg; biotin, 0.24 mg; D-pantothenic acid, 18.355 mg; niacin, 6 mg; folic acid, 0.14 mg. ² Mineral premix provided the following nutrients per kg of diet: Fe, 70 mg; Mn, 7.5 mg; Zn, 60 mg; Cu, 7.5 mg; I, 1 mg; Co, 0.13 mg; Se, 0.20 mg.

2.3. Measurements of Laying Performance and Egg Quality

The feed intake of each replicate was measured at the beginning and end of the experiment to calculate the average daily feed consumption per bird. Egg production and egg weight were recorded daily and used to calculate the egg mass. Feed-to-egg ratio was determined as total feed consumption divided by egg mass. During the final three days of the trial, six unbroken eggs were sampled from each replicate. The TSS QCR reflectometer (Technical Services and Supplies, York, UK) was used to measure eggshell color. A digital egg tester (DET-6000, Nabel, Kyoto, Japan) was employed to assess Haugh unit, eggshell strength, eggshell thickness (excluding the shell membrane) and yolk color score.

2.4. Intestinal Sample Collection

At 28 days, one hen per replicate was chosen at random and euthanized using a carbon dioxide overdose. Laying hens that were not used for sampling in this study were not culled but raised at the KU research farm. The small intestine and paired ceca were sampled immediately after euthanasia. The jejunal segment was also sampled for immune cell subpopulation analysis. For histological analysis, an approximately 1 cm mid-segment of the ileum was fixed in 10% neutral buffered formalin solution for 48 h. The remaining distal ileal segment was processed for the measurement of antioxidant/immune markers. A pair of ceca were kept on ice and processed to assess the concentrations of short-chain fatty acids (SCFAs), which serve as an important indicator of microbial fermentation activity in the cecal digesta, on the day of sampling.

2.5. Measurements of Ileal Histology

Hematoxylin and eosin staining was applied to 5 µm paraffin sections, per standard protocols [22]. The mucosal layer was evaluated under a light microscope (Olympus BX43, Tokyo, Japan) and captured with a digital camera (eXcope T500, DIXI Science, Daejeon, Republic of Korea). Villus height and crypt depth were quantified by analyzing ten properly sectioned villi and crypts. The villus height-to-crypt depth ratio was subsequently determined.

2.6. Measurements of Antioxidant and Immune Markers in Ileal Mucosa

The ileal mucosa samples were obtained by carefully scraping the distal ileal segment with a tissue-culture scraper. Next, the samples were then centrifuged at $1000 \times g$ for 10 min at 4 °C and stored at −20 °C until analysis. The activities of glutathione peroxidase, superoxide dismutase, and catalase, and the concentrations of malondialdehyde and secretory immunoglobulin A were determined using commercial assay kits. These analyses were conducted using the following commercially available kits in order: EnzyChrom™ Glutathione Peroxidase Assay Kit, EnzyChrom™ Superoxide Dismutase Assay Kit (BioAssay Systems, Hayward, CA, USA), OxiSelect™ Catalase Activity Assay Kit, OxiSelect™ TBARS Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA), and Chicken IgA ELISA Kit (Bethyl Laboratories, Montgomery, TX, USA). The concentrations of total protein in ileal mucosa samples were measured with Bradford assay reagent (Sigma-Aldrich, St. Louis, MO, USA).

2.7. Measurements of Short-Chain Fatty Acids (SCFAs) in Cecal Digesta

Approximately 1 g of cecal digesta was homogenized in 4 mL of ice-cold distilled water and added with 0.05 mL of saturated HgCl_2 , 1.00 mL of 25% H_3PO_4 , and 0.20 mL of 2% pivalic acid. The mixture was then centrifuged at $1000 \times g$ for 20 min at 4 °C. Following centrifugation, 1 mL of the supernatant was collected for SCFA analysis using gas chromatography (6890 Series GC System, HP, Palo Alto, CA, USA), as previously detailed [23].

2.8. Measurements of Jejunal Immune Cell Subpopulation Using Flow Cytometer

Immune cells in jejunal mucosa samples were isolated by density gradient centrifugation, as outlined by [24], and analyzed using the FACS Canto II system (BD, Franklin Lakes, NJ, USA). Viable cells were identified by excluding dead cells through live/dead fixable dead cell stain (Thermo Fisher Scientific, Waltham, MA, USA). For staining, the anti-chicken antibodies, including anti-CD3 (CT-3), anti-CD4 (CT-4), anti-CD8a (CT-8), anti-TCR (TCR-1), anti-MHC II (2G11), anti-Bu-1 (AV20), and anti-Monocyte/Macrophage (KUL01), were utilized (Southern Biotech, Birmingham, AL, USA).

2.9. Statistical Analysis

Data was analyzed with SAS software (SAS 9.4, SAS Institute Inc., Cary, NC, USA). PROC UNIVARIATE (SAS 9.4, SAS Institute Inc., USA) was applied to test for outliers and normal distribution across all variables. All experimental results are expressed as means and pooled standard error of the means. Each experimental unit consisted of three adjacent cages. Student's *t*-test was employed to compare between dietary treatments. Statistical significance was declared at $p < 0.05$.

3. Results

3.1. Laying Performance and Egg Quality

Dietary supplementation with *L. paracasei* NSMJ27 did not affect ($p > 0.05$) laying performance, including feed intake, egg production, egg mass, egg weight, or feed-to-egg ratio in laying hens (Table 2). No supplemental effects of *L. paracasei* were observed on yolk color, Haugh unit, eggshell strength, or eggshell thickness. Eggshell color tended to be darker ($p = 0.057$) in laying hens fed diets containing *L. paracasei* vs. control diets (Table 3).

Table 2. Effect of dietary *Lacticaseibacillus paracasei* strain NSMJ27 on laying performance in laying hens ¹.

Items	CON	NSMJ27	Pooled SEM	<i>p</i> -Value
Feed intake, g/d/bird	99.69	98.04	1.246	0.206
Egg production, %	84.52	85.80	1.139	0.289
Egg weight, g/egg	61.18	61.58	0.267	0.164
Egg mass, g/d	52.24	52.33	1.262	0.944
Feed-to-egg ratio, kg/kg	1.897	1.861	0.025	0.174

¹ CON, control diet; NSJM27, CON + 2.5×10^9 CFU/kg *L. paracasei* subsp. *paracasei* NSMJ27; SEM, standard error of the means.

Table 3. Effect of dietary *Lacticaseibacillus paracasei* strain NSMJ27 on egg quality in laying hens ¹.

Items	CON	NSMJ27	Pooled SEM	<i>p</i> -Value
Yolk color	7.517	7.670	0.162	0.368
Haugh unit	87.13	87.21	0.716	0.912
Eggshell strength, kg/cm ²	4.870	4.479	0.220	0.106
Eggshell thickness, mm	0.390	0.383	0.011	0.596
Eggshell color, unit	24.98	23.22	0.843	0.057

¹ CON, control diet; NSJM27, CON + 2.5×10^9 CFU/kg *L. paracasei* subsp. *paracasei* NSMJ27; SEM, standard error of the means.

3.2. Ileal Histology

Dietary *L. paracasei* NSMJ27 did not affect ($p > 0.05$) ileal morphology, including villus height, crypt depth, and villus height-to-crypt depth ratio (Table 4). Nonetheless, probiotic-fed laying hens had a higher villus height: crypt depth ratio ($p = 0.067$) by on average 15.2% compared with the control diet-fed treatment.

Table 4. Effect of dietary *Lacticaseibacillus paracasei* strain NSMJ27 on ileal histology in laying hens ¹.

Items	CON	NSMJ27	Pooled SEM	<i>p</i> -Value
Villus height (VH), μ m	824.7	814.0	51.30	0.839
Crypt depth (CD), μ m	134.4	127.1	4.159	0.114
VH:CD ratio	5.543	6.386	0.419	0.067

¹ CON, control diet; NSJM27, CON + 2.5×10^9 CFU/kg *L. paracasei* subsp. *paracasei* NSMJ27; SEM, standard error of the means.

3.3. Short-Chain Fatty Acids in Cecal Digesta

Acetate was the dominant short-chain fatty acid, followed by propionate and butyrate in cecal digesta (Table 5). Dietary *L. paracasei* NSMJ27 did not affect the relative percentages of acetate, propionate, isobutyrate, valerate, branched-chain fatty acids or short-chain fatty acids. However, dietary *L. paracasei* NSMJ27 increased ($p = 0.016$) the relative percentage of butyrate by on average 19.7% but decreased ($p = 0.057$) that of isovalerate in cecal digesta by on average 26.0% compared with the control group.

Table 5. Effect of dietary *Lacticaseibacillus paracasei* strain NSMJ27 on the relative percentage (% of total) of short-chain fatty acids in cecal digesta of laying hens ¹.

Items	CON	NSMJ27	Pooled SEM	<i>p</i> -Value
Acetate	60.18	58.93	3.189	0.701
Propionate	16.62	16.52	1.712	0.951
Isobutyrate	3.613	4.157	1.183	0.653
Butyrate	10.90 ^b	13.05 ^a	0.782	0.016

Table 5. Cont.

Items	CON	NSMJ27	Pooled SEM	p-Value
Isovalerate	3.735	2.763	0.469	0.057
Valerate	4.948	4.582	0.497	0.474
BCFA	12.30	11.50	1.660	0.640
SCFA	87.70	88.50	1.660	0.640

¹ CON, control diet; NSJM27, CON + 2.5×10^9 CFU/kg *L. paracasei* subsp. *paracasei* NSMJ27; SEM, standard error of the means; SCFAs, short-chain fatty acids (acetate + propionate + butyrate); BCFA, branched-chain fatty acids (isobutyrate + isovalerate + valerate). ^{a,b} Values having a different superscript within a row differ significantly ($p < 0.05$).

3.4. Antioxidant and Immune Markers in Ileal Mucosa

Dietary *L. paracasei* NSMJ27 increased ($p = 0.048$) the superoxide dismutase activity in ileal mucosa compared with the control group. However, no effect by dietary *L. paracasei* was noted ($p > 0.05$) in the activities of catalase and glutathione peroxidase, or the contents of malondialdehyde of ileal mucosa. In addition to antioxidant markers, secretory immunoglobulin A, a key marker of the gut mucosal immune system, was assessed to evaluate the effect of dietary *L. paracasei* on gut health. It was found that dietary *L. paracasei* did not affect ($p = 0.622$) the concentration of secretory immunoglobulin A in the ileal mucosa of laying hens (Table 6).

Table 6. Effect of dietary *Lactacaseibacillus paracasei* strain NSMJ27 on antioxidant and immune markers of ileal mucosa in laying hens ¹.

Items	CON	NSMJ27	Pooled SEM	p-Value
SOD, U/mg protein	1.494 ^b	1.921 ^a	0.192	0.048
MDA, nmol/mg protein	3.281	2.690	0.717	0.424
CAT, U/mg protein	8.567	8.690	0.978	0.902
GPX, U/mg protein	0.164	0.142	0.043	0.608
sIgA, ug/mg protein	34.30	38.96	9.185	0.622

¹ CON, control diet; NSJM27, CON + 2.5×10^9 CFU/kg *L. paracasei* subsp. *paracasei* NSMJ27; SEM, standard error of the means; SOD, superoxide dismutase; MDA, malondialdehyde; CAT, catalase; GPX, glutathione peroxidase; sIgA, secretory immunoglobulin A. ^{a,b} Values having a different superscript within a row differ significantly ($p < 0.05$).

3.5. Jejunal Immune Cell Subpopulations

Dietary *L. paracasei* NSMJ27 did not affect ($p > 0.05$) the subpopulations of immune cells expressing the macrophage, B cell and T cell surface markers (CD4+, CD8+, and TCR $\gamma\delta$ + T cells) (Figure 1) in jejunal mucosa samples.

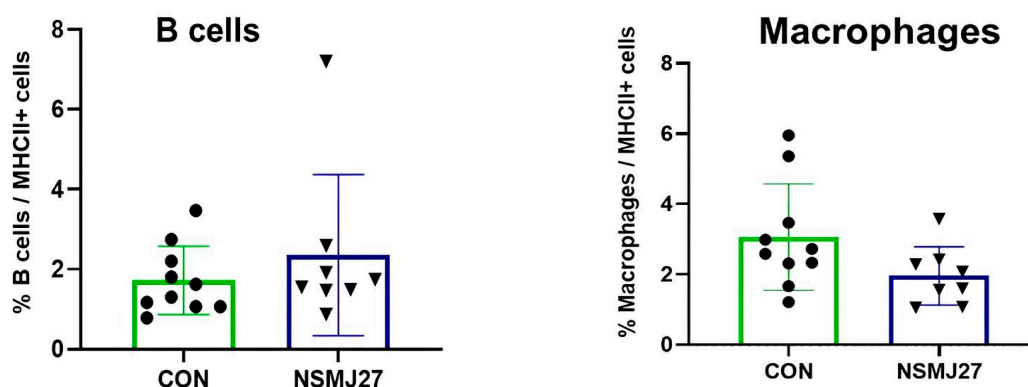


Figure 1. Cont.

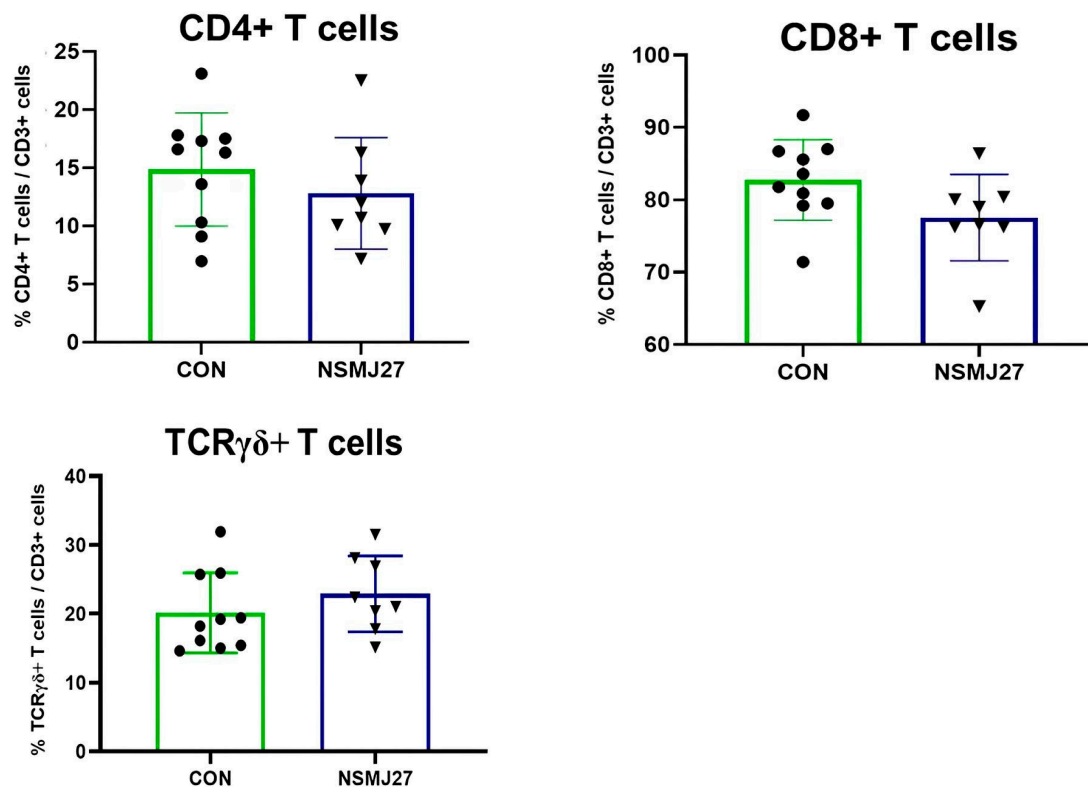


Figure 1. Effect of dietary *Lactocaseibacillus paracasei* NSMJ27 on jejunal lymphocyte subpopulations in laying hens. The percentages of cells expressing the indicated surface markers (B cells, macrophages, CD4, CD8, TCR $\gamma\delta$) assessed by flow cytometry. Filled circle and triangle symbols indicate individual data per treatment. Data is presented as mean and standard deviation. Abbreviations: CON, control diet; NSMJ27, diet supplemented with 2.5×10^9 CFU/kg *L. paracasei* subsp. *paracasei* NSMJ27.

4. Discussion

The interplay between small intestinal morphology, antioxidant defense systems, and mucosal immunity is integral in optimizing nutrient utilization, promoting growth, and enhancing egg production in laying hens, which allowed us to analyze ileal morphology and gut health indicators, including ileal antioxidant/immune markers and cecal short-chain fatty acids. It is clear from this study that dietary *L. paracasei* NSMJ27, recently isolated from fermented foods with probiotic potentials, did not affect the laying performance or egg quality parameters of aged laying hens. These findings are consistent with previous studies in laying hens fed combinations of *L. paracasei* [25] and *Bacillus subtilis* [26]. A trend toward darker eggshell color was observed in the *L. paracasei* diet treatment, which may be attributed to a healthier intestinal environment leading to enhanced absorption of mineral or pigment precursors necessary for eggshell pigmentation [27–29]. Indeed, dietary *L. paracasei* NSMJ27 displayed beneficial properties in improving gut health (ileal villus height: crypt depth ratio, butyrate in cecal digesta and superoxide dismutase activity in ileal mucosa) in aged laying hens. It should be pointed out, however, that the feeding trial lasted 4 weeks in this study, although the effectiveness (e.g., antioxidant/immune markers) of dietary probiotics, if any, can be disclosed in laying hens. In this sense, caution is needed to conclude the lack of probiotic effect on laying performance and egg quality, which may need a longer duration of feeding (i.e., more than 12 weeks).

The beneficial role of dietary probiotics on ileal morphology (e.g., villus height: crypt depth ratio) has been well reported in broilers [30,31] and laying hens [32,33]. It is postulated that probiotic-mediated improvement in ileal morphology is enacted by either balancing gut microbiota [34] or by augmenting local immunity [3], leading to improved

nutrient absorption. In this study, laying performance (e.g., feed-to-egg ratio), secretory immunoglobulin A in ileal mucosa, and jejunal immune cell subpopulations including macrophages, B cells and T cells were not altered by dietary *L. paracasei*. Thus, it is likely that dietary *L. paracasei* NSMJ27 might improve ileal villus height: crypt depth ratio via balancing the composition of gut microbiota in ileal digesta. Indeed, we found that dietary *L. paracasei* NSMJ27 altered short-chain fatty acids (increased butyrate and decreased isovalerate in cecal digesta), which are regulated by beneficial gut microbiota. In line with our study, a higher villus height: crypt depth ratio was noted in laying hens fed *L. salivarius* SNK-6 [35], *L. crispatus* [36], *Bacillus coagulans* [37], and *B. subtilis* [38].

Our results demonstrated that dietary *L. paracasei* markedly enhanced the superoxide dismutase activity in the ileal mucosa of aged laying hens, suggesting a significant improvement in local antioxidant defense mechanisms. Our finding is consistent with previous reports [14,39,40] which documented the increased antioxidant enzyme activities in various tissues of poultry following supplementation with *Lactobacillus* and *Lactococcus* species. This enhanced antioxidant status likely stems from *L. paracasei*'s capacity to modulate mucosal immune responses [21,41] or produce bioactive metabolites [42] that reduce reactive oxygen species damaging epithelial tissues. The present study also demonstrated that dietary *L. paracasei* NSMJ27 enhanced the relative percentage of butyrate in the cecal digesta of laying hens. In addition to the function of short-chain fatty acids (i.e., butyrate) as the primary energy source for colonocytes, it is known that they can contribute to improving gut health and to modulating immune responses [43]. Kim et al. [18] reported that laying hens fed a diet containing *L. paracasei* NSMJ56 had an altered cecal microbial community via increasing short-chain fatty acid-producing bacteria including *Flintibacter*, *Diella*, and *Coprotherobacter*. In addition, Thananimit et al. [44] identified specific *L. paracasei* strains exhibiting high butyrate production potential. Apart from the increase in butyrate concentrations by dietary *L. paracasei* NSMJ27, it lowered the relative percentage of isovalerate in cecal digesta. It is known that branched-chain fatty acids, including isovalerate, are produced from bacteria-mediated fermentation on undigested proteins [45]. This is significant, as elevated concentrations of branched-chain fatty acids are associated with increased protein degradation and potential pathogenic bacterial activity [45,46]. Tentatively, our findings demonstrate that *L. paracasei* NSMJ27 favors a beneficial shift in the cecal bacterial community, increasing carbohydrate fermentation but lowering protein fermentation in the ceca. Taken together, our findings demonstrate that *L. paracasei* NSMJ27 supplementation strengthens intestinal health through a coordinated enhancement of local antioxidant defenses, the promotion of beneficial bacterial metabolism, and a reduction in metabolic byproducts associated with unfavorable microbiota composition.

5. Conclusions

In summary, *L. paracasei* NSMJ27 supplementation did not affect laying performance but improved ileal villus height: crypt depth ratio, superoxide dismutase in ileal mucosa, and the relative percentage of butyrate in cecal digesta. This study shows that dietary *L. paracasei* NSMJ27 can be used as a feed additive to enhance the gut health of aged laying hens.

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