Effects of *Diutina rugosa* SD-17 on growth performance, intestine morphology, and immune status of chickens

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ABSTRACT Probiotics are considered the preferred alternatives to antibiotics for growth promotion and disease prevention. Previous studies have confirmed that *Diutina rugosa* SD-17 has the potential as a probiotic. We evaluated the probiotic effect of *D. rugosa* SD-17 on 360 one-day-old Hy-line brown chickens that were divided into 2 groups, and each group contained 6 replicate pens with 30 birds per pen. The chickens were fed with basal diet supplemented with or without *D. rugosa* SD-17, and the effects of *D. rugosa* SD-17 on growth performance, intestine morphology, and immune status were assessed. Body weight was significantly improved from week 3 to 6 ($P < 0.05$), and the feed conversion ratio was significantly improved in weeks 1 and 2 ($P < 0.001$). The length of the duodenum was lengthened significantly in week 3 ($P < 0.05$), and supplementation of *D. rugosa* SD-17 significantly increased villus height and villus height to crypt depth ratio in the ileum in week 3 ($P < 0.05$). Expressions of tight-junction-related genes of zonula occludens-2 and occludin in the ileum were significantly increased ($P < 0.05$). The proliferation capacity of blood lymphocytes stimulated by concanavalin A was significantly enhanced ($P < 0.05$), and the proportion of helper T (**Th**) cells increased significantly ($P < 0.05$). Expressions of Th1 cell markers IL-2 and interferon (IFN)-γ and immune-related genes of IL-1β, transforming growth factor-β, and IFN-γ in ileum were significantly increased ($P < 0.05$). These results indicated that *D. rugosa* SD-17 improved the growth and regulated immunity of chickens, and could be optimized for use as a feed additive for livestock and poultry.

Key words: *Diutina rugosa* SD-17, growth performance, intestine morphology, immunity

INTRODUCTION

In recent years, the safety of animal-derived food has been questioned due to the abuse of antibiotics (Yeping et al., 2014). To eliminate safety hazards at the source, some feed additives, such as antibiotics and hormones, which seriously threaten food safety, have been banned from animal feed (Castanon, 2007; Millet and Maertens, 2011; Mohsina et al., 2015). Since antibiotics and hormones can prevent disease and promote the growth of livestock and poultry (Dibner and Richards, 2005), prohibition of their use will inevitably lead to economic losses in animal husbandry. It is an urgent problem to find feed additives that are harmless to livestock and poultry and can resist disease and promote growth, instead of antibiotics and hormones (Seal et al., 2013). Studies have shown that probiotics are good substitutes for antibiotics or hormones (Reid and Friendship, 2002; Kritas and Morrison, 2005).

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Probiotics are defined as live microorganisms that confer a health benefit on the host when administered in adequate amounts (FAO/WHO, 2006). It is worth noting that probiotic effects are strain specific (Araya et al., 2002). Ramos et al. (2013) found that a total of 234 *Lactobacillus* isolates from Brazilian food products were strain specific and 3 isolates exhibited potential probiotic properties. Our previous study also reached a similar conclusion. *Diutina rugosa* SD-17 isolated from chicken feces had significant probiotic performance in vitro, while the type strain *D. rugosa* ATCC10571 had no potential as a probiotic (Wang et al., 2019).

Diutina rugosa is a hemiascus, asporogenic, and nonpathogenic yeast. Although it is found extensively in the environment, it is mainly used for the industrial production of lipases (Benjamin and Pandey, 1998; Hasan et al., 2006). So far, there are no reports on the use of *D. rugosa* for livestock production. Our previous studies have shown that *D. rugosa* SD-17 has strong adaptability in the simulated gastrointestinal environment in vitro and can be temporarily retained in the intestine (Wang et al., 2019), but these results only indicated that *D. rugosa* SD-17 has potential as a probiotic. In the present study, we determined the effects of

Table 1. Ingredients and nutrient composition of diets.

Ingredient (%)		Energy and nutrient content	
Corn	61.7	Metabolizable energy (Kcal/kg)	3,236
Wheat bran	4.5	Crude protein (%)	16.96
Bean pulp	24	Crude fiber (%)	3.12
Fish meal	2	Methionine (%)	0.49
Rapeseed meal	5	Lysine (%)	0.88
Calcium phosphate	1.3		
Limestone	1.2		
Salt	0.3		

D. rugosa SD-17 on growth performance, intestinal morphology, and the immune system of chickens to demonstrate its probiotic effects and clarify its probiotic mechanisms.

MATERIALS AND METHODS

Animal Ethics Statement

All animal experimental protocols were approved by the Shandong Agricultural University Animal Care and Use Committee (SDAUA-2017–38).

Experimental Design and Animal Management

A total of 360 one-day-old Hy-Line Brown chickens were purchased from Dongyue Breeding Poultry Co. Ltd. (Shandong, China). The chickens were randomly divided into 2 experimental groups: group 1, control diet; and group 2, *D. rugosa* SD-17-supplemented diet. Each group contained 6 replicate pens with 30 birds per pen, and animals were fed a balanced, unmedicated corn and soybean meal-based mash diet that contained either 0 (control) or 1×10^7 CFU/g *D. rugosa* SD-17. *Diutina rugosa* SD-17 used in the experiments was provided by our laboratory. The composition of the basal diet and nutrient levels are presented in Table 1. The room temperature was maintained at 33°C during the first 5 D and then gradually decreased by 3°C each week until it reached 24°C. Chickens had free access to feed and water, and were housed in wire cages, received continuous light for the first 24 h, and were then maintained under 23 L: 1 D for the remainder of the study.

Growth Performance

Chickens and feed intake were group weighed by cage at 1, 2, 3, 4, 5, and 6 wk of age. ADG, ADFI, and feed conversion ratio (**FCR**) were calculated for each period and for the overall experiment. These performance parameters were corrected according to mortality.

Intestinal Morphology

At 2, 3, and 4 wk, 6 birds from each replicate pen were randomly selected and killed. The gastrointestinal tract was removed and the small intestine was divided into 3 parts: duodenum (from the gizzard outlet to the end of the pancreatic loop); jejunum (from the pancreatic loop to Meckel's diverticulum); and ileum (from Meckel's diverticulum to the ileo-ceco-colic junction), and the length of each section was measured. At 3 wk, 1-cm-long segments were taken from the center of each part and fixed in 10% buffered formalin. Tissues were serial dehydrated by transferring through alcohols with increasing concentrations and embedded in paraffin. Tissue sections were cut by a microtome and stained with hematoxylin and eosin. Images were obtained using a microscope, and histomorphometric measurement was performed by using Photoshop software. A total of 10 well-oriented, intact villi and crypts were randomly selected in duplicate from each tissue sample and the average of 20 values obtained for each chicken was taken.

Immune Organ Index

Six birds from each replicate pen were randomly selected and killed by cervical dislocation at 2, 3, and 4 wk. The bursa of Fabricius, thymus, and spleen were dissected out, surface moisture was removed with a filter paper, and pathological changes to the organ surface were recorded. Finally, the organs were weighed and the immune organ index was calculated.

Blood Lymphocyte Proliferation Assay and Subpopulations

At 3 wk, 6 birds from each group were randomly selected, weighed, and wing venous blood was collected in a 1-mL injection syringe containing 4% sodium citrate. Peripheral blood lymphocytes (**PBLs**) were isolated using the lymphocyte separation medium (Solarbio, Beijing, China) (Fair et al., 2008). The PBLs were seeded into 96-well microtiter plates (Costar, Cambridge, MA) at 10^5 cells per well in sextuplicate, with or without concanavalin A (Con A) (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO). The plates were incubated in 5% CO_2 at 37°C for 66 h. Cell Counting Kit-8 reagent (Solarbio) was added to each well, followed by incubation for 2 h at 37°C, and optical density at 450 nm (OD_{450}) was measured using an automated microplate reader (Biotek Instruments, Winooski, VT). The results of the lymphocyte proliferation assay were expressed as stimulation indices, which were presented as the ratio of absorbance of stimulated cells to that of unstimulated cells from the same chicken. To measure the percentage of $CD4^+$ and $CD8^+$ cells, the isolated PBLs were incubated with mouse anti-chicken CD3-PE/Cy5 (Southern
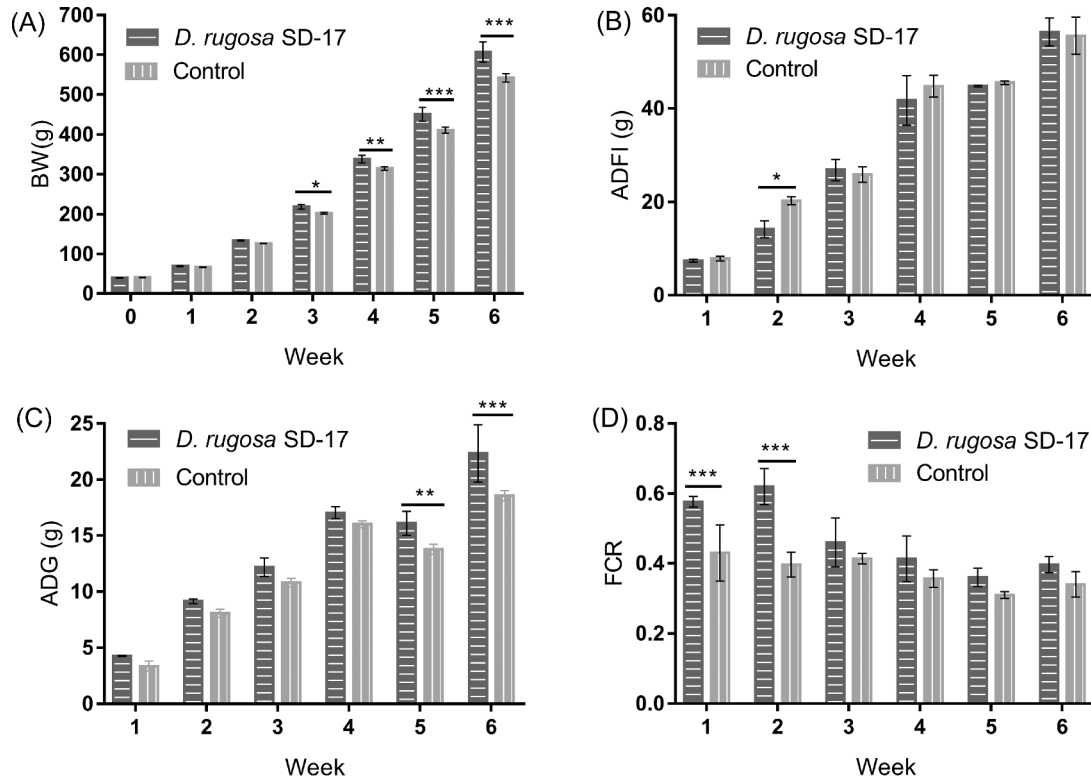
Figure 1. Effects of *Diutina rugosa* SD-17 on growth performance in chickens. Effects of *D. rugosa* SD-17 on (A) BW; (B) ADFI; (C) ADG; and (D) feed conversion ratio (FCR). Control = birds fed a basal diet; *D. rugosa* SD-17 = birds fed a basal diet supplemented with 1×10^7 cfu/kg *D. rugosa* SD-17. Each mean represents 10 replicates.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Biotech, Birmingham, AL) and mouse anti-chicken CD8a- FITC (Southern Biotech) or mouse anti-chicken CD4-FITC (Southern Biotech) at 4°C for 20 min and analyzed using flow cytometry.

Detection of Cytokines

At 3 wk, 2-cm-long segments were taken from the center of the ileum and quickly transferred to a mortar precooled with liquid nitrogen, and ground to powder with a pestle. The sample homogenate amount of RNAiso Plus (Takara, Dalian, China) was added to the mortar for RNA extraction. Blood lymphocytes were directly treated with RNAiso Plus for RNA extraction. cDNA synthesis was conducted using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara). Quantitative real-time polymerase chain reaction (**qPCR**) was performed using an Applied Biosystems 7500 Real-Time PCR System (Invitrogen Life Technologies, Carlsbad, CA) using TB Green Premix Ex Taq (Takara) with appropriate primers (Supplementary data). Glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) was used as an endogenous control for data normalization. Based on expression of target genes normalized to *GAPDH* levels, relative $^{\triangle\triangle}$Ct values were calculated and the results presented as fold change compared to controls.

Statistical Analysis

The data were evaluated by unpaired *t*-tests using Graph Pad Prism version 3.00. The data are expressed as the mean ± standard error.

RESULTS

Growth Performance

None of the chickens in the *D. rugosa* SD-17 group died or showed obvious pathological changes. Two and one chicken died in the control group on days 6 and 7, respectively, although no obvious pathological changes were seen macroscopically appearance or through pathological examination. Birds fed *D. rugosa* SD-17 diets had significantly greater body weight than those in the control group at weeks 3 ($P < 0.05$), 4 ($P < 0.01$), 5 ($P < 0.001$), and 6 ($P < 0.001$) (Figure 1A). ADFI decreased significantly at week 2 ($P < 0.05$; Figure 1B). In addition, ADG increased significantly at weeks 5 and 6 ($P < 0.01$ and $P < 0.001$; Figure 1C). FCR was significantly improved at weeks 1 and 2 ($P < 0.001$; Figure 1D).

Intestinal Morphology

Intestinal Development By visual and histopathological observation, no bleeding, injury, and other

Table 2. Change in intestinal length with or without *D. rugosa* SD-17 supplementation.[1]

Intestine	Age	Control	*D. rugosa* SD-17	*P*-value
Duodenum	2	14.5 ± 0.58	16 ± 1.10	0.313
	3	14.0 ± 0.63	16.78 ± 0.97	0.023
	4	16.67 ± 0.82	19.44 ± 1.13	0.023
Jejunum	2	31.75 ± 1.71	29.17 ± 2.79	0.084
	3	38.33 ± 3.62	39.44 ± 3.36	0.359
	4	38.67 ± 1.97	40.89 ± 3.41	0.069
Ileum	2	21.5 ± 3.00	23.00 ± 2.53	0.313
	3	27.00 ± 1.58	28.78 ± 1.99	0.167
	4	32.83 ± 3.06	32.89 ± 2.15	0.963

Results are reported as the means ± SD.
[1]Each mean represents the data of 6 birds.

Table 3. Effects of level of supplementation of *D. rugosa* SD-17 on small intestinal morphology in chickens.

	Control	*D. rugosa* SD-17	*P*-value
Duodenum			
VH[1] (μm)	1262.2 ± 77.74	1294.1 ± 15.86	0.245
CD[2] (μm)	318.2 ± 11.85	306.3 ± 21.02	0.661
VH/CD ratio	3.97 ± 0.15	4.23 ± 0.24	0.129
Jejunum			
VH (μm)	828.3 ± 28.52	845.7 ± 32.11	0.520
CD (μm)	119.2 ± 8.49	123.83 ± 6.17	0.865
VH/CD ratio	6.95 ± 0.25	6.83 ± 0.09	0.472
Ileum			
VH (μm)	409.6 ± 29.34	477.0 ± 50.72	0.019
CD (μm)	103.2 ± 7.76	103.0 ± 17.39	0.993
VH/CD ratio	3.97 ± 0.04[a]	4.66 ± 0.30[b]	0.001

Results are reported as the means ± SD.
[1]Villus height.
[2]Crypt depth.

lesions were found in the intestinal tract of the chickens in the 2 groups. The duodenal lengths of the *D. rugosa* SD-17 group were significantly longer than that of the control group at 3 and 4 wk ($P < 0.05$, Table 2). There was no significant difference in the length of jejunum and ileum between the groups at 2, 3, and 4 wk ($P > 0.05$). The *D. rugosa* SD-17 group had normal intestinal structure; the highest villi were in the duodenum followed by lower villi in the jejunum, and the lowest being in the ileum (Table 3). The intestinal villi were slim and finger-shaped and the intestinal mucosa revealed no histopathological changes in chickens from both groups. At 3 wk, *D. rugosa* SD-17 significantly increased villus height and villus height to crypt depth ratio in the ileum ($P < 0.05$). However, crypt depth was not affected by dietary supplementation of *D. rugosa* SD-17 ($P > 0.05$).

Expression of Tight-Junction-Related Genes We detected changes in the mRNA expression of tight-junction-related genes in the duodenum, ileum, and cecum. Expression of zonula occludens (**ZO**)-2 and occludin was significantly upregulated by 4.19-fold ($P < 0.05$, Figure 2B) and 3.55-fold ($P < 0.05$, Figure 2H), respectively, in the ileum. Expression of claudin-1 was significantly reduced by 2.13-fold ($P < 0.05$, Figure 2E). Expression of occludin in the duodenum was significantly upregulated by 2.53-fold ($P < 0.05$, Figure 2H).

Expressions of the other of genes of ZO-1, β-catenin, E-cadherin, claudin-1, and claudin-5 were not significantly changed ($P > 0.05$, Figure 2A, C, D, F, and G).

Changes in the Immune System

Immune Organ Index The immune organ indices of the thymus, spleen, and bursa of Fabricius of the *D. rugosa* SD-17 group at weeks 2, 3, and 4 showed no significant difference compared with those of the control group.

Subpopulations and Proliferation of Blood Lymphocytes The percentage of CD3+CD4+ Th cells in the *D. rugosa* SD-17 group was significantly higher than in the control group ($P < 0.05$), but there was no significant change in CD3+CD8+ cytotoxic T cells ($P > 0.05$; Figure 3A and B). Lymphocyte proliferation in response to ConA is shown in Figure 3C. The *D. rugosa* SD-17 group displayed significantly higher lymphocyte proliferation in response to ConA than that of the control group ($P < 0.05$).

Expression of Cytokines in T-Lymphocytes Changes in the mRNA expressions of cytokines in T lymphocytes are shown in Figure 3D. The expressions of Th1 cell markers IL-2 and interferon (**IFN**)-γ were significantly upregulated by 9.96- and 10.62- fold ($P < 0.05$), respectively, while expressions of the Th2 cell markers IL-4 and IL-5 were not significantly changed. Expressions of IL-1β, IL-12, tumor necrosis factor (**TNF**)-α, and transforming growth factor (**TGF**)-β were upregulated by 3.52-, 4.72-, 2.64-, and 6.98-fold, respectively ($P < 0.05$), but expressions of IL-6 and IL-10 were not obviously changed.

Expression of Immune-Related Genes in the Ileum The expression of immune-related genes in the ileum was detected. Expressions of IL-1β, TGF-β, and IFN-γ were significantly upregulated by 2.02-, 5.19-, and 36.11-fold, respectively ($P < 0.05$), but expressions of IL-2, IL-6, IL-8, IL-10, IL-12, IL-18, TNF-α, and inducible nitric oxide synthase were not significantly changed (Figure 3E).

DISCUSSION

Evidence has been presented that probiotics promote the growth of poultry, but the exact mechanism through which this occurs is considered to be complex (Bai et al., 2017; Forte et al., 2018), and could include the production of nutrients, stimulation of intestinal development, and/or fermentation of the feed. In this study, the basal diet supplemented with *D. rugosa* SD-17 significantly increased BW and FCR, and significantly decreased ADFI, which may be related to *D. rugosa* lipase. *Diutina rugosa* is a strong lipase producer (Tan et al., 2003), and *D. rugosa* lipase has excellent traits, such as stability at high temperatures and activity at a broad range of pH values (Treichel et al., 2010; Geoffry and Achur, 2018). Therefore, *D. rugosa* SD-17 can break down lipids to produce fatty acids in poor
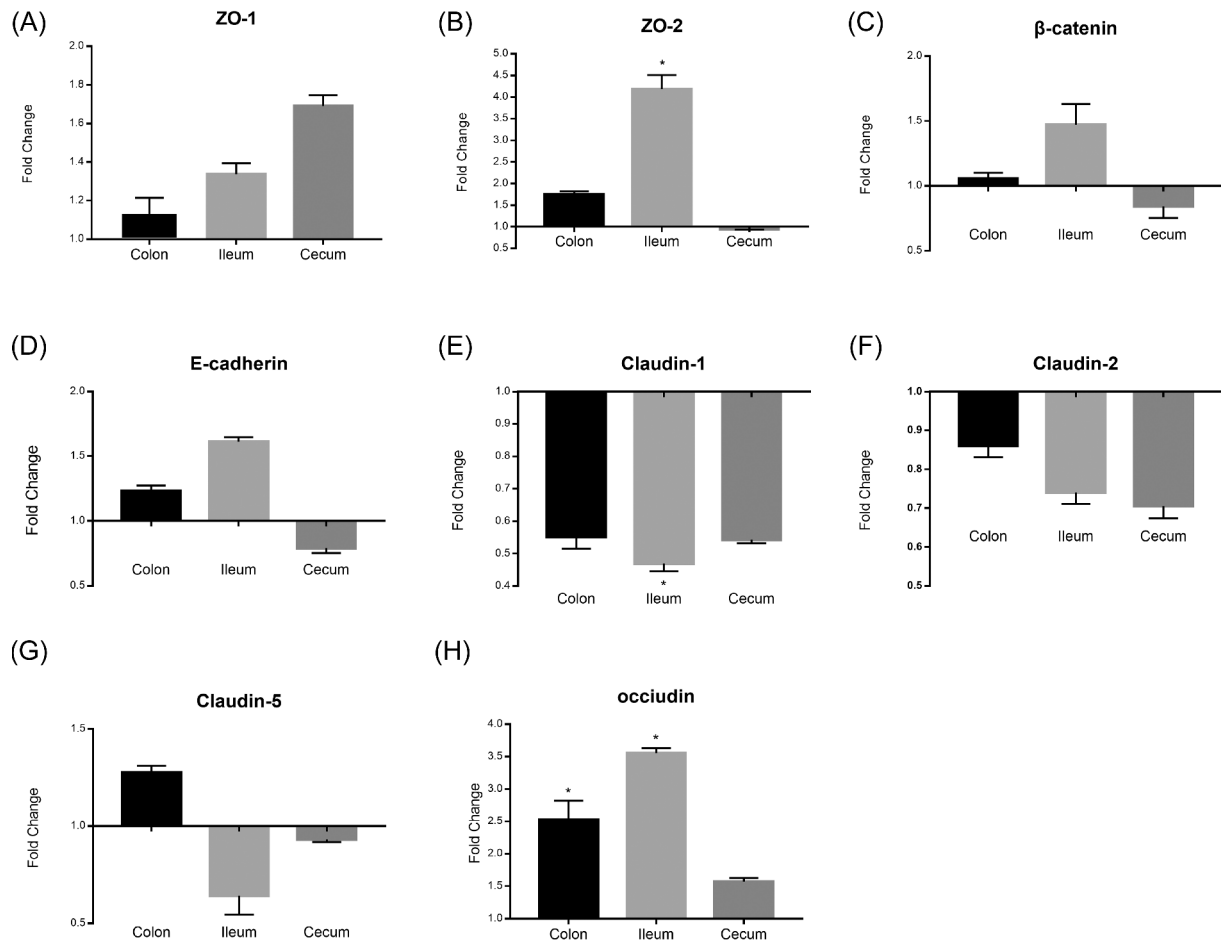
Figure 2. Expression of tight-junction-related genes in the colon, ileum, and cecum of chickens. (A) Zonula occludens (ZO)-1, (B) ZO-2, (C) β-catenin, (D) E-cadherin, (E) claudin-1, (F) claudin-3, (G) claudin-5, and (H) occludin. The fold change represents gene expression in diet with *D. rugosa* SD-17 compared to that of the controls. Bars represent the mean $\pm$ SD of 3 independent experiments, $*P < 0.05$.

gastrointestinal environments. Short-chain fatty acids can regulate intestinal mucosal homeostasis and modulate neuronal excitability (Soret et al., 2010; Ríos-Covián et al., 2016), thereby improving gastrointestinal motility and promoting digestion and absorption of feed. Previous studies have shown that β-glucan is an immunomodulator that can be extracted from the cell walls of yeast, which can be directly absorbed and utilized by the gut and can significantly improve growth performance and enhance the innate immune system (Rouhier et al., 1995; Tian et al., 2016). In our previous study, we found that the content of glucan in *D. rugosa* SD-17 was higher than that of another type strain, which may be an important reason for the growth promotion effect of *D. rugosa* SD-17 in chickens (Chae et al., 2006).

Improving the intestinal morphology is another way by which probiotics play a probiotic role, and the height of the villi in the small intestine is strongly associated with their intestinal absorption (Yamauchi, 2002). Many studies have shown that probiotics can promote normal development of the intestinal villi, including *Bacillus subtilis* (Sen et al., 2012), *Lactobacillus* (Awad et al., 2009), and *Enterococcus faecalis* (Cao et al.,

2013). Similarly, *D. rugosa* SD-17 increased the height of ileal villi in the present study. Crypt depth is associated with infection, which represents the ability of intestinal epithelial cells to renew and repair themselves in the event of infection. Cao et al. (2013) have reported that *Escherichia coli* K88 increased jejunal crypt depth by infecting broiler chickens, and *Enterococcus faecium* reduced infection and jejunal crypt depth. It has been reported that probiotics have no significant effect on the depth of the crypt under healthy conditions (Awad et al., 2009). Likewise, in our study *D. rugosa* SD-17 had no effect on the crypt depth of duodenum, jejunum, and ileum. This suggested that *D. rugosa* SD-17 did not invade the intestinal epithelial cells and cause intestinal infection. In addition, *D. rugosa* SD-17 promoted duodenal development and the length of the duodenum increased significantly, which was conducive to improving digestion and absorption. Tight junctions are an important part of the epithelial barrier, and probiotics have been shown to promote intestinal barrier integrity in vitro and in vivo (Yu et al., 2012; Wang et al., 2018). Tight junctions are complex protein structures composed of transmembrane proteins (Ma et al., 2018). Treatment of epithelial cells with *E. coli* Nissle 1917
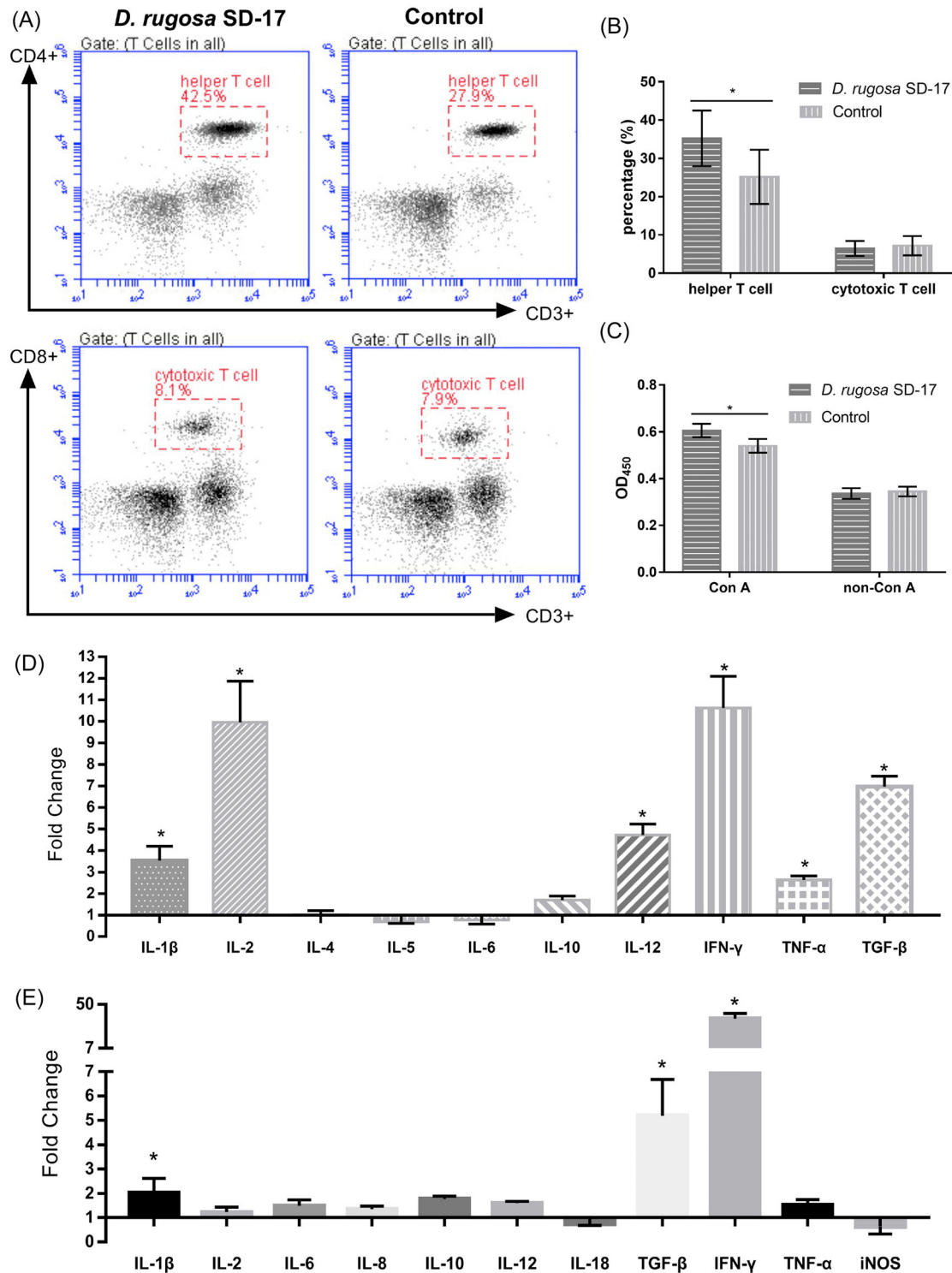
Figure 3. (A) T-lymphocyte sorting through fluorescence activated cell sorting (FACS). Each dot in the plot indicates a single cell. (B) The percentages of CD4$^+$ and CD8$^+$ cells in the blood. (C) Blood lymphocyte proliferation responses to ConA. (D) Expression of immune-related genes in blood lymphocytes of chickens. (E) Expression of immune-related genes in ileum of chickens. Con A, concanavalin A; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; iNOS, inducible nitric oxide synthase. Each bar represents the mean $\pm$ SD (n = 3) and each bird sample was analyzed in triplicate. *$P < 0.05$.

leads to increased expression of ZO-2 tight junction protein and redistribution of ZO-2 from the cytosol to cell boundaries (Ukena et al., 2007; Zyrek et al., 2007). In addition, Ewaschuk et al. (2008) and Anderson et al. (2010) found that treatment of epithelial cells with the probiotic product VSL#3 increased transcription of occludin and ZO-1 gene expression while reducing claudin gene expression. A similar effect was observed in our study; supplementation of *D. rugosa* SD-17 increased transcription of ZO-2 and occludin and decreased

transcription of claudin-1, suggesting that *D. rugosa* SD-17 altered the expression of tight junction proteins to regulate intestinal barrier function.

T lymphocytes and their functions are important components of the immune system (Burnette and Weichselbaum, 2013; Yahfoufi et al., 2018). Previous studies have shown that probiotics promote proliferation of lymphocytes (Kirjavainen et al., 1999; Bujalance et al., 2007). Our study confirmed this. *D. rugosa* SD-17 promoted the proliferation of T lymphocytes, which indicated that it stimulated nonspecific immunity. Th cells and cytotoxic T cells are the main types of blood T lymphocytes, and their proportion directly reflects the level of specific immunity (Zhu et al., 2010; Kaech and Cui, 2012). Studies have confirmed that Th cells play central roles in the function of the immune system by orchestrating immune responses against a wide variety of pathogenic microorganisms (Biedermann et al., 2004; Zhou et al., 2008; Sant et al., 2018). In our study, *D. rugosa* SD-17 increased the proportion of Th cells but had no effect on cytotoxic T cells. This showed that *D. rugosa* SD-17 could help improve resistance to external microorganisms. Th1 cells represent one of the different subsets of the heterogeneous Th $CD4^+$ population, and these cells are characterized by the production of IL-2, IFN-γ, and lymphotoxin as signature cytokines (Annunziato and Romagnani, 2016), whereas these lymphokines are not detectably expressed in Th2 clones. Conversely, only Th2 clones synthesize detectable amounts of IL-4 and IL-5 (Mosmann and Coffman, 1989). It has been shown that probiotics stimulate the secretion of cytokines by Th1 cells (Ghadimi et al., 2008). Similar conclusions were reached in the present study. The expressions of IL-2 and IFN-γ at the transcript levels were significantly increased but expression of IL-4 was not significantly affected. These results indicated that *D. rugosa* SD-17 changed the direction of lymphocyte differentiation toward Th1 cells. It has been shown that probiotics stimulate immune cells to release proinflammatory cytokines, including IL-1β, TNF-α, and IFN-γ (Tsai et al., 2010). The expressions of IL-1β and IFN-γ were significantly increased in this study. In addition, the expression of TGF-β was significantly increased, which suggests that TGF-β is a regulator of cytokines and plays an important role in regulating immunity (Wahl, 2007; Kubiczkova et al., 2012).

In conclusion, supplementation with *D. rugosa* SD-17 improved growth performance, intestinal morphology, and immunity in chickens. Because this study was conducted on healthy chickens, whether *D. rugosa* SD-17 would also show beneficial effects on sick chickens is worthy of further study.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Table S1. Gene-specific sequences primers used in real-time quantitative PCR.

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