Animal Science Journal

Animal Science Journal (2017) **88,** 1204–1211 doi:10.1111/asj.12757

ORIGINAL ARTICLE

Oral administration of *Saccharomyces boulardii* alters duodenal morphology, enzymatic activity and cytokine production response in broiler chickens

Yajing SUN,[1] Imran Rashid RAJPUT,[1,2] Muhammad Asif ARAIN,[1,3] Yanfei LI[1] and Dost Muhammad BALOCH[3]

[1]*College of Sciences, Shantou University,* [2]*College of Animal Sciences and Technology, North West Agriculture and Forestry University, China; and* [3]*Faculty of Veterinary and Animal Sciences, Lasbela University of Agriculture, Water and Marine Sciences, Uthal, Balochistan, Pakistan*

ABSTRACT

The present study evaluated the effects of *Saccharomyces boulardii* on duodenal digestive enzymes, morphology and cytokine induction response in broiler chicken. A total of 200 birds were allotted into two groups (n = 100) and each group divided into five replications (n = 20). The control group was fed basal diet in addition to antibiotic (virginiamycin 20 mg/kg), and treatment group received (1×10^8 colony-forming units/kg feed) *S. boulardii* in addition to basal diet lasting for 72 days. The results compared to control group revealed that adenosine triphosphatase, gamma glutamyl transpeptidase, lipase and trypsin activities were higher, while, no significant improvement was observed in amylase activities in the duodenum of the treatment group. Moreover, morphological findings showed that villus height, width and number of goblet cells markedly increased. Additionally, transmission electron microscopy visualized that villus height, width and structural condensation significantly increased in the treatment group. The immunohistological observations showed increased numbers of immunoglobulin A (IgA)-positive cells in the duodenum of the treatment group. Meanwhile, cytokine production levels of tumor necrosis factor-α, interleukin (IL)-10, transforming growth factor-β and secretory IgA markedly increased, and IL-6 statistically remained unchanged as compared to the control group. These findings illustrated that initial contact of *S. boulardii* to the duodenum has significant impact in improving enzymatic activity, intestinal morphology and cytokine response in broiler chicken.

Key words: *broiler, cytokines, duodenum, intestine, S. boulardii.*

INTRODUCTION

Oral administration of probiotics has been utilized to improve intestinal health by maintaining the morphology, enzymatic activity, normal microflora and induction of cytokines (Wageha *et al.* 2008). These functions of probiotics play a critical role in the reinforcement of the intestinal mucosal barrier against invading pathogens (Fioramonti *et al.* 2003). However, several clinical trials have been performed to assess the efficacy of various probiotics to observe their effects on intestinal morphological structure and immunity (Szajewska *et al.* 2007). Moreover, different modes of action of probiotics have been described, that they modulate the commensal bacteria to generate resistance by modulating intestinal structure (Bernet-Camard *et al.* 1997) and they participate in nutrient utilization by competitive binding to sites on epithelial cells (Sherman *et al.* 2005). Generally several antibiotics, bacitracin, virginiamycin and others, are commonly used in poultry as growth-promoting, but these antibiotics have severe hazardous effects on human health. An increased demand for poultry and the ban on the use of antibiotics has caused attention to switch to non-antibiotic strategies. However, probiotics produce antibacterial substances against harmful organisms to protect the host epithelial cells (Varcoe *et al.* 2003), and interact with the epithelial linings to strengthen the tight junction against invading microorganisms, by improving the mucosal immune response (Gedek 1999).

Another study suggested the beneficial effects of probiotics through production of cytokines and improvement in the innate immunity of the host (Sherman *et al.* 2005), but response may vary from species to species (Rajput *et al.* 2013a).

Correspondence: Imran Rashid Rajput, College of Science, Shantou University, Shantou, Guangdong, China. (Email: drimranrajput@gmail.com)
Received 3 September 2016; accepted for publication 28 October 2016.

Yeast as a probiotic is considered to be a useful organism that produces several beneficial effects, and *Saccharomyces boulardii* is one of the recently known probiotics which improve the intestinal immunity by modulating the ultrastructure (Rajput *et al.* 2013b). This probiotic acts as a shuttle liberating effect to several factors during intestinal transit, to improve host immunity, digestion and absorption of nutrients. (Rajput *et al.* 2013c). It secretes polyamines, mainly spermine and spermidine, during its intestinal transit that regulate gene expression and protein synthesis. (Buts & De Keyser 2006). Some bacteria may recognize binding sites in such molecules as if they are present on the mucosal surface, and the intestinal colonization by pathogenic bacteria is thus reduced (Choi *et al.* 1994). Thus, there are lower chances of infectious incidence, and the functions of secretion, digestion and absorption of nutrients can be appropriately performed by the mucosa (Iji & Tivey 1998). Although the exact mechanism by which *S. boulardii* might exerts beneficial effects in different parts of the small intestine is unclear (Sougioultzis *et al.* 2006), a recent study was conducted by Rajput *et al.* (2013a), who reported that *S. boulardii* modulates the jejunal and ileal morphology ultrastructure by modulating cytokines production. However, the question remains whether the duodenum is the first interaction by probiotics to modulate the response in broiler chickens, and it is unclear in the literature that *S. boulardii* may have effects on enzymatic activity, morphology and cytokine production in the duodenum of broiler chickens. Therefore, previous findings inspired us to investigate the effects of *S. boulardii* on morphology, enzymatic activity and cytokine production in the duodenum of broiler chickens.

MATERIALS AND METHODS

Probiotics preparation

Saccharomyces boulardii was isolated and identified by the Institute of Animal Nutrition and Feed Science, Zhejiang University. The yeast was cultured in yeast peptone dextrose (YPD) in aerobic conditions at 30°C for 24 . Centrifugation was applied at 6000 × *g* for 5 min to separate the yeast pellets. The yeast was washed twice with phosphate-buffered saline (PBS, pH 7.3), and pellets were added into basal diet (Table 1) and maintained (1×10^8 colony-forming units (cfu)/kg).

Experimental design

A total of 200 day-old Sanhuang broilers (Chinese cross breed) were randomly allotted into two groups, and each group consisted of 100 birds with five replications ($n = 20$). Basal diet (Table 1) was fed to the control group (Ctr) in addition to antibiotics (virginiamycin 20 mg/kg,), whereas, birds in the experimental group were fed basal diet in addition to *S. boulardii* (1×10^8 cfu/kg: *Sb* group) and the feeding trail lasted for 72 days.

Sample collection

After feeding lasted for 72 days, broilers were sacrificed as per recommendations of Shantou University Animal Centre (SUAC) and the first part of the small intestine, the duodenum, was collected and opened longitudinally with a micro-scalpel aseptically. Then they were transferred into separate sterilized tubes containing formalin (10%; Sigma, Shanghai, China) to process for histological examination. To determine the enzymatic activities, samples were placed in PBS (pH 7.4) and ultrasonic treatment applied for 4 min in order to separate the gut contents from the gastrointestinal tissue.

Histological examination

To investigate the effects of *S. boulardii* on the histological structure of the small intestine, the method as described by Rajput *et al.* (2013b) was applied. Briefly, the duodenal sections of 1 cm thickness were collected and washed in sodium chloride solution (0.85% Sigma), fixed in buffered formalin (10%) overnight and embedded in paraffin. Afterwards 2 μm diameter tissue sections were mounted on glass slides and stained. Further, tissues were cleared, hydrated and stained with Alcian Blue solution (pH 2.5) for 30 min. After washing with water for 10 min, slides were oxidized in periodic acid (5 g/L) for 5 min, then rinsed in lukewarm tap water for 10 min and stained in Coleman's Schiff reagent as a

Table 1 Composition and nutrition of the basal experimental diet (%)

Composition	1-36 days	37-72 days
Corn	55.90	61.60
Soybean meal	31.00	27.00
Wheat shorts	3.00	4.00
Imported fish meal	5.00	2.00
Rapeseed oil	1.50	2.00
Salt	0.30	0.30
Dicalcium phosphate	1.20	1.00
Limestone	1.00	1.00
DL-Methionine	0.10	
Lysine		0.10
Premix	1.00	1.00
Total	100.00	100.00
Nutrient		
Metabolizable energy (MJ/kg)	12.78	13.05
Crude protein	22.86	19.14
Lysine	1.07	0.98
Methionine + Cysteine	0.86	0.72
Ash	7.38	6.41
Ca	0.93	0.91
Total phosphorus	0.64	0.56

Premix compound: each kg contained: vitamin A, 7000 IU; vitamin D_3, 2500 IU; vitamin E, 30 mg; vitamin K_3, 1 mg; vitamin B_1, 1.5 mg; vitamin B_2, 4 mg; vitamin B_6, 2 mg; vitamin B_{12}, 0.02 mg; niacin, 30 mg; folic acid, 0.55 mg; pantothenic acid, 10 mg; biotin, 0.16 mg; choline chloride, 400 mg; Cu, 20 mg; Fe, 70 mg; Mn, 100 mg; Zn, 70 mg; I, 0.4 mg; Se, 0.5 mg; virginiamycin 20 mg/kg.

counterstain for 10 min. The slides were photographed under a Nikon microscope (Nikon Corp., Tokyo, Japan), and measurement was performed using image software, Cast Image System (Visiopharm, Horsholm, Denmark).

Transmission electron microscopy (TEM)

Tissues from the duodenum were randomly selected ($n = 8$), and approximately 2–3 mm longitudinal sections were prepared aseptically and transferred into sterilized round bottom tubes containing sufficient quantities of fixative solution (3% glutaraldehyde, pH 7.4) for 12 h at room temperature. The next, tissues were rinsed and subsequently processed for TEM in routine processing operations by the Medical College Shantou University P.R China.

Enzymatic activities analysis

The duodenal homogenates were collected after ultrasonic treatment using PBS (pH 7.4), and centrifuged at 5000 × *g* for 25 min at 4°C, then the supernatant was collected for enzyme assays. In brief, the activities of Na^+ K^+ adenosine triphosphatase (ATP), gamma glutamyl transpeptidase (γGT), α-amylase (AMS), trypsin (TPS), lipase (LPS), were determined by a microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA), by using diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the method described by the manufacturers.

Immunohistochemistry of duodenal immunoglobulin A (IgA) cells

Duodenum samples were serially embedded in paraffin and cut into 6 μm lengths using a Leica CM3050 cryostat and mounted on polylysine-coated glass slides. The sections were immunostained by the indirect immunoperoxidase method (Khan *et al.* 1997). In brief, endogenous peroxidase was inhibited with 3% H_2O_2 in methanol for 7–10 min, and thrice rinsed in PBS for 5 min. The sections were incubated in 0.01 mol/L citrate-buffered solution (pH 6.0) at 95°C for 20 min for antigen retrieval, then allowed to cool at room temperature and rinsed thrice in PBS. After that, the sections were treated with 5% fetal calf serum in PBS to block non-specific binding at room temperature for 30 min. The excess solution was shaken off and the slides around the tissue section were blotted. The sections were incubated with goat anti-chicken IgA antibody (1:900; Bethyl Laboratories, Montgomery, TX, USA) at 4°C for 12 h, and then washed three times in PBS for 15 min, followed by incubation with mouse anti-goat IgG conjugated horseradish peroxidase (1:250; Sigma-Aldrich) at 37°C for 1 h. After the sections were rinsed with PBS for 10 min, the reactions were made visible with metal-enhanced diaminobenzidine (DAB; Sigma, Shanghai, China). A moist chamber was used to perform all of incubations. Control staining was carried out simultaneously in which the first antibody was replaced with PBS. No specific staining was found in the control.

Determination of cytokines by ELISA

The ELISA was performed to determine the interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-10, transforming growth factor (TGF)-β (Genorise Scientific Inc, Paoli, PA, USA) cytokines and secretory IgA (sIgA) (Komabiotech, Ltd., Seoul, Korea) immunoglobulin, as previously described by Rajput *et al.* (2013a). Briefly, polyclonal goat anti-chicken IL-6, TNF-α, IL-10 TGF-β and sIgA, antibodies were applied as capturing antibodies, biotinylated polyclonal goat anti-chicken IL-6, TNF-α, IL-10 TGF-β and sIgA antibodies as detecting antibodies. Streptavidin-RP and Tetramethylbenzidine Solution (TMBS) were used as color indicators and subsequent color reaction was stopped with 2 mol/L sulfuric acid. Absorbance of each well was measured at (450 nm) wavelength, right after incubation at (37°C for 10 min).

Statistical analyses

Data was analyzed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA) and expressed as mean ± standard deviation (SD). The variation between groups was assessed by *t*-test statistical significance. Statistical significance of the results was calculated at $P < 0.05$.

RESULTS

Live body weight and internal organs weight

The findings showed ($P < 0.05$) increased (Fig. 1) live body weight of broilers in the *Sb* group as compared to the Ctr group. Meanwhile, the bursa and thymus also

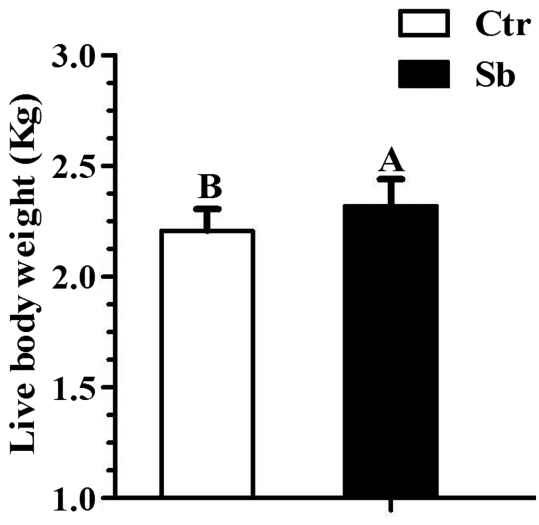

Figure 1 Effects of oral administration of probiotics on live body weight of control (Ctr) and *Saccharomyces boulardii* (Sb) groups. Values are mean ± SD. Different letters show significant difference ($P < 0.05$) between the groups.

revealed higher weight ($P < 0.05$) in the *Sb* group, as compared with the Ctr group (Table 2).

Determination of digestive enzymes activity

In the present study, specific digestive enzyme activities were assessed (Fig. 2) in the duodenum of broiler chickens. After administration of *S. boulardii* the results were compared to a control group; it showed that ATP, γGT, LP and TPS activities were significantly higher in the duodenum of the *Sb* group, while no significant improvement was observed in AMS activities in the duodenum of treatment group as compared with the Ctr group.

Histological examination

Our findings illustrated that *S. boulardii* supplementation (Fig. 3) improved the villus height and width and numbers of goblet cells also increased ($P < 0.05$) in the duodenum and crypts of Langerhans areas remained unchanged as compared with the Ctr group.

TEM

TEM was performed to confirm the histological findings of the villus (Fig. 4). The villi structure appeared very clear and visualized the increased villus height, width and condensation in the duodenum of the *Sb* group as compared with the Ctr group.

Immunohistochemistry

The immunohistological observations showed (Fig. 5) obvious improvement in the intestinal segment and morphological examination revealed the increased number of IgA-positive cells ($P < 0.05$) in the duodenum of the probiotics group in comparison with the control group.

Cytokines analysis by ELISA

The cytokine production levels in the duodenum are shown in (Fig. 6). In the present finding, the *Sb* group markedly increased the production of TNF-α, IL-10, TGF-β and sIgA. However, IL-6 levels numerically improved and statistically remained unchanged as compared to the control group.

Table 2 Internal organ weight percentage between control (Ctr) and *Saccharomyces boulardii* (*Sb*) groups

Internal organs	Ctr	*Sb*
Liver (%)	1.92 ± 0.14	2.00 ± 0.11
Spleen (%)	0.23 ± 0.02	0.24 ± 0.03
Bursa (%)	0.17 ± 0.05[b]	0.25 ± 0.04[a]
Thymus (%)	0.29 ± 0.07[b]	0.41 ± 0.07[a]

Effects of dietary inclusion of *S. boulardii* on internal organs of broilers, between Ctr and *Sb*. Values are mean ± SD. [a,b]Rows with different superscript letters show significant differences ($P < 0.05$) between the groups.

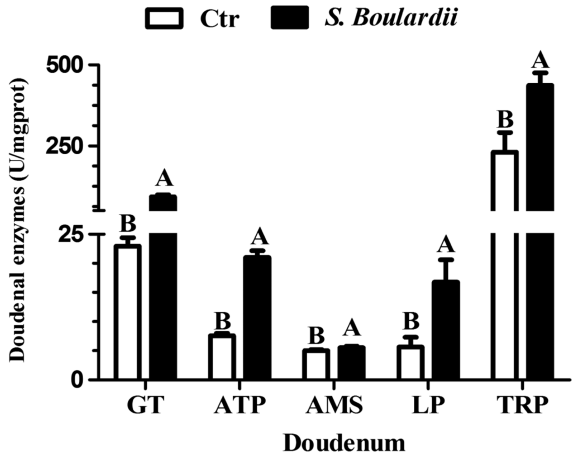

Figure 2 Effect of *Saccharomyces boulardii* on digestive enzyme activity of duodenum and data is represented as (mean ± SD), $P < 0.05$ was considered to be statistically significant. Different letters show statistical differences.

DISCUSSION

Intestinal surface is one of the most important sites which is vulnerable to micro-organisms causing infection. This surface has immunological and non-immunological mechanisms to protect itself against invading organisms (Mestecky & Mcghee 1992), while, immune cells known as mobile forces, that is macrophage and dendritic cells in between and beneath the epithelium surface, play an important role in the local immunological defense. Therefore, we studied the effects of *S. boulardii* on alteration of digestive enzymes, morphological structure and cytokine production response in broiler chickens.

In the present study we observed that ATP and γGT activities significantly increased in the duodenum, followed by LP and TRP functions in comparison to the control group. Similarly, the beneficial effects of probiotics to enhance digestive enzyme activities were reported by (Sanders 1993). Moreover, findings by Chevalier *et al.* (1999), illustrated that prokaryotes (yeast) enhance the activities of γGT and ATP in the intestine. Another study showed improvements in jejunal and ileal digestive enzyme activity after administration of *S. boulardii* (Rajput *et al.* 2013a). These digestive enzymes play a crucial role in metabolizing the feed and/or food. The polypeptide chains of feed and food (or both) in the intestine are converted into simple amino acids. This process is mainly achieved by a brush border enzyme γGT (Smith *et al.* 1991), in the intestine. The catalysis process decomposes adenosine triphosphate into adenosine diphosphate (ADP), and free phosphate ions that release the energy. This energy is utilized by the enzymes to drive other chemical reactions and to help in the transportation of amino acids (Cotgreave & Schuppe-Koistinen 1994). Thus, our findings of enzymatic activity in the duodenum describe that *S. boulardii*

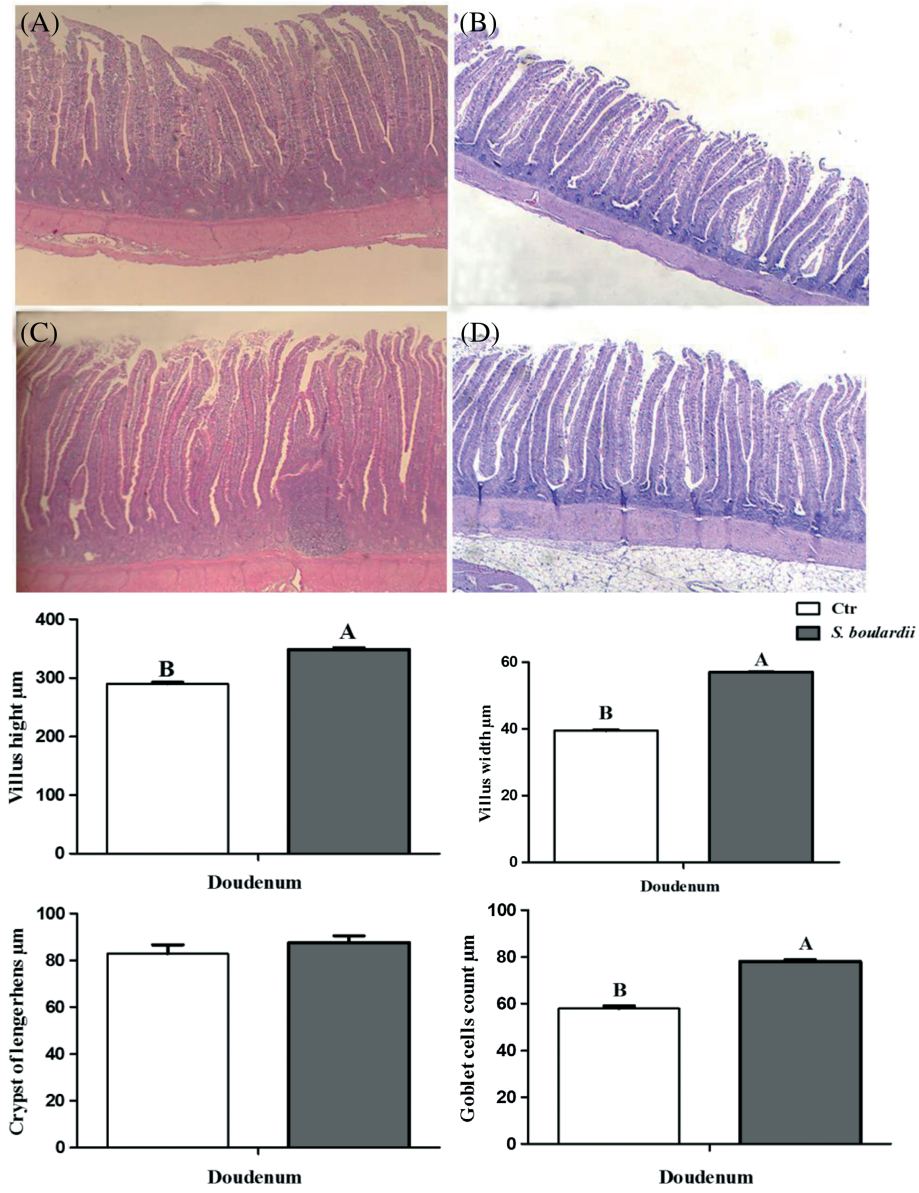

Figure 3 Hematoxylin and eosin (A, C) and periodic acid-Schiff staining (B, D) of duodenum. A, B showing control group, (Ctr); C, D, *Saccharomyces boulardii* group. Staining visualized the villus structure, height, width and crypts of Langerhans at magnification of ×100. Graph bar values are mean ± SD. Different letters show significant differences between groups ($P < 0.05$), $n = 8$.

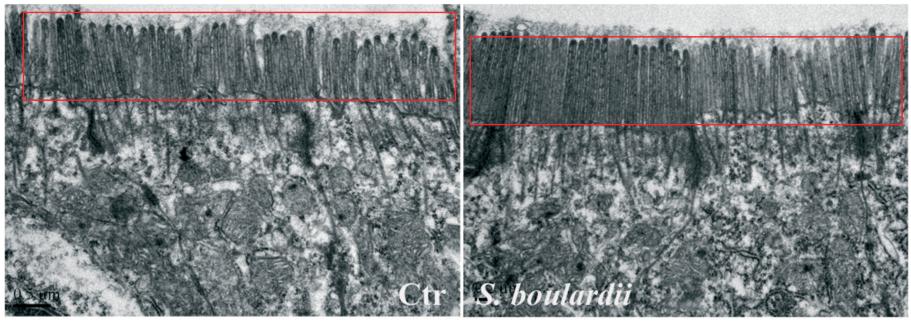

Figure 4 Electron micrograph images of the duodenal tissue from broiler, control (Ctr), and *Saccharomyces boulardii* (*S. boulardii*). Top of the villus and end points can be observed clearly in the micrograph.

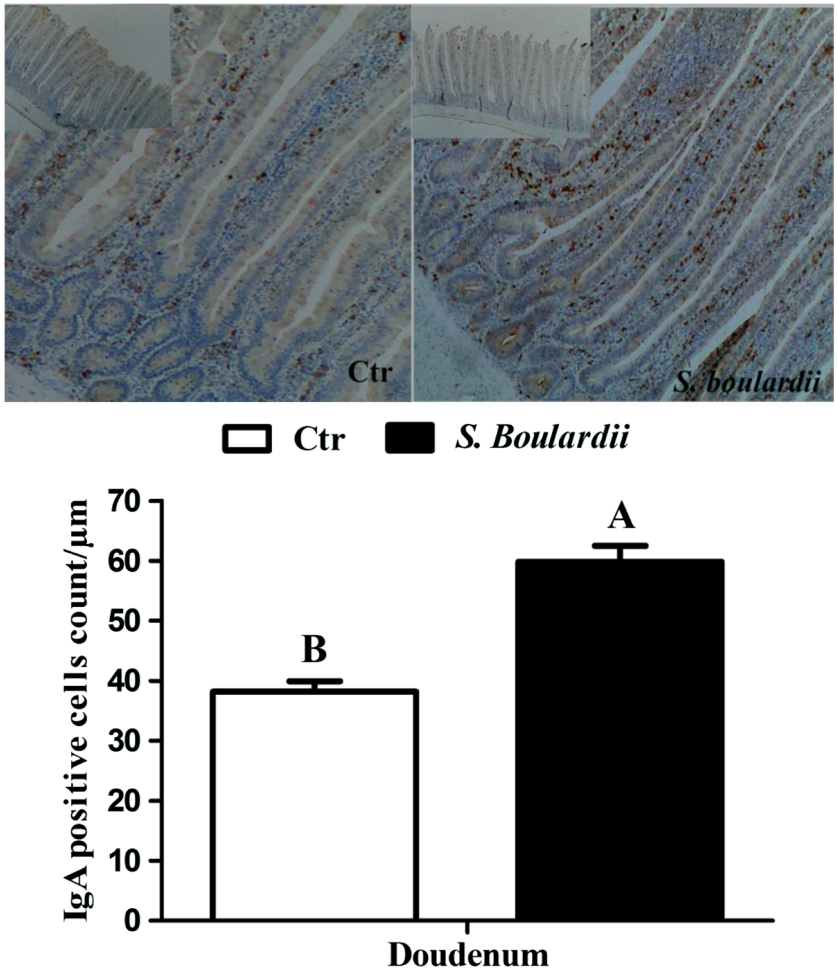

Figure 5 Immunoglobulin A (IgA)+ cells in duodenum section, indicated the IgA+ cells between control (Ctr) and *Saccharomyces boulardii* (*S. boulardii*), groups at magnification of (×40). Brown color stained in the lumen of the villi and arrows indicate IgA-positive cells. Values are mean ± SD, and different letters show significant difference ($P < 0.05$), $n = 8$.

might be supporting protein metabolism in broiler chickens.

Villi are only the most obvious feature of the mucosa which contains a dynamic, self-renewing population of epithelial cells. These epithelial cells originate from crypts of Langerhans and migrate along the villus surface upward to the villus tip and disperse in lumen of villi; moreover the active mitosis induces the longer size of villi in response to providing nutrition to the animals (Potten 1998). In this study, oral administration of *S. boulardii* provided the evidence that villus height, width and number of goblet cells significantly increased in the duodenum, and further confirmation was displayed by TEM. Similarly, *Lactobacillus reuteri* application in broiler diets showed long increased height of villi in the intestine (Dunham *et al.* 1993), along with this, endothelial cell proliferation improvement was noted in rats after probiotics treatment (Ichikawa *et al.* 1999). Structure and architecture of the intestinal villi also regulate major functions, increase nutrients absorption, homeostasis and defense against invading organisms. We also found morphological alteration in villus height and architecture improvement in the duodenum of broiler chickens.

IgA secreting cells are often used to evaluate intestinal mucosal immunity (Zhang *et al.* 2007). However, a fraction of IgA antibodies is also generated in T cells by an independent manner (Macpherson *et al.* 2000). In the present study similar results were observed that *S. boulardii* could improve the IgA positive cell numbers and sIgA secretion levels in the duodenum of broiler chickens. However, TGF-β signaling plays a central role in the induction of mucosal IgA; TGF- β stimulates the isotype switch to IgA secretion by LPS-stimulation (Ehrhardt *et al.* 1992; Kim & Kagnoff 1990). Moreover, IL-6 and TGF-β promote class switching of B cells to produce IgA (Cerutti & Rescigno 2008), that activate and proliferate by interactions with local environmental T cells; additionally, unknown structural components of Gram-positive bacteria are also responsible for elicitation of intestinal IgA antibodies (Bos *et al.* 2001). sIgA is the important effector molecule to protect mucosal surfaces

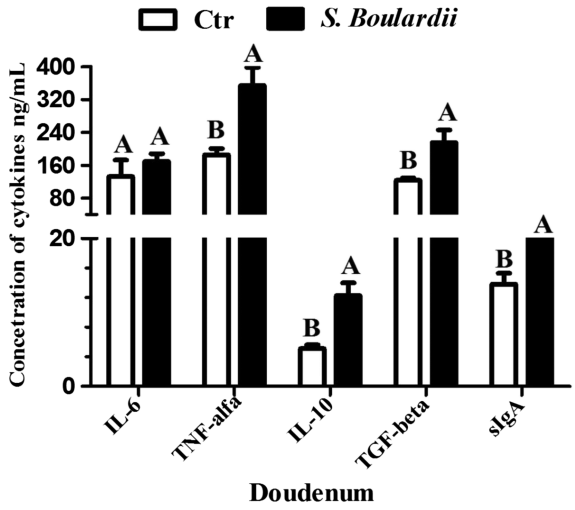

Figure 6 Effects of orally administered probiotics (1×10^8 colony-forming units/g), *Saccharomyces Boulardii* (*S. boulardii)* duodenum cytokines interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-10 transforming growth factor (TGF)-β and secretory immunoglobulin A (sIgA) secretion levels were determined by ELISA, and fixed optical density (450 nm) was applied. The results are represented as means ± SD ($n = 8$). Different letters show significant difference ($P < 0.05$), between groups.

from B cells in the laminal propria of the intestine. It has the mechanisms for antigen neutralization, prevention of microbial attachment to epithelial surface, elimination of excessive antigen load, and the overall maintenance of mucosal homeostasis (Johansson *et al.* 1999; Haruki *et al.* 2008). Additionally, application of probiotics stimulates immune cells and subsequently cytokine production (Lammers *et al.* 2003). So the increased production of sIgA and IgA+ cells may be due to improved levels of IL-6 and TGF-β in the duodenum. It was appealing to know that *S. boulardii* induced IgA+ cells and sIgA secretion in the duodenum of broiler chickens.

Inflammatory cytokines play a critical role in eliciting protective immunity allied to T-helper cells (Rajput & Li 2012) and cytokine production that may be dependent of probiotic species (Atarashi *et al.* 2011). In accordance to current data, *S. boulardii* significantly increased the production of TNF-α, IL-10, TGF-β and sIgA, while IL-6 numerically improved. Another study reported that probiotics improved TNF-α and IL-10 production levels in the colonic tissues (Jeon *et al.* 2012) and *Clostridium* species have been shown to stimulate TGF-β in the intestine through the epithelial cells (Huang *et al.* 2011). Previously, Gitter *et al.* (2000), and Yan *et al.* (2006) illustrated that TNF-α causes an improvement in intestinal permeability by maintaining the homeostasis of Na^+ and K^+ channels of cell membrane (Kayamuro *et al.* 2009), and TNF-α is a well recognized innate immune responses mediator (Trevejo *et al.* 2001). Initiation of inflammatory cytokines is characterized by the primary secretion of pro-inflammatory cytokines (Eckmann & Kagnoff 2001). Afterwards, regulation of inflammatory cytokines is directed by the release of IL-10, therefore increasing and decreasing responses of Th1 cells are noted (Groux & Powrie 1999).Therefore, during infection, TGF-β production levels are initially up- and shortly down-regulated (Coburn *et al.* 2007). Current data provided information that probiotic strain *S. boulardii* promotes epithelial cell cytokine production levels to induce mucosal immunity in broiler chickens.

Conclusion

Conclusively, the present study delineated that *S. boulardii* is an effective probiotic to enhance enzyme activities, morphological improvement and induce cytokine production in the duodenum as a first contact part of the small intestine of broiler chickens. This probiotic could be used as a feed additive in the poultry industry.

ACKNOWLEDGMENTS

This study was supported by grants obtained from the national project Specialized Research Fund for the Higher Education and Research.

REFERENCES

Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T. 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* **331**, 337–341.

Bernet-Camard MF, Lievin V, Brassart D, Neeser JR, Servin AL, Hudault S. 1997. The human *Lactobacillus acidophilus* strain LA1 secretes a non-bacteriocin antibacterial substance(s) active in vitro and in vivo. *Applied and Environmental Microbiology* **63**, 2747–2753.

Bos NA, Jiangand HQ, Cebra JJ. 2001. T cell control of the gut IgA response against commensal bacteria. *Gut* **48**, 762–764.

Buts JP, De Keyser N. 2006. Effects of Saccharomyces boulardii on intestinal mucosa. *Digestive Diseases and Sciences* **51**, 1485–1492.

Cerutti A, Rescigno M. 2008. The biology of intestinal immunoglobulin a responses. *Immunity* **28**, 740–750.

Chevalier C, Thiberge JM, Ferrero RL, Labigne A. 1999. Essential role of *Helicobacter pylori*gamma-glutamyltranspeptidase for the colonization of the gastric mucosa of mice. *Molecular Microbiology* **31**, 1359–1372.

Choi KH, Namkung H, Paik IK. 1994. Effects of dietary fructooligosaccharides on suppression of intestinal colonization of Salmonella typhimurium in broiler chickens. *Korean Journal of Animal Science* **36**, 71–284.

Coburn B, Grassl GA, Finlay BB. 2007. Salmonella, the host and disease: a brief review. *Immunology and Cell Biology* **85**, 112–118.

Cotgreave IA, Schuppe-Koistinen I. 1994. A role for gamma-glutamyl transpeptidase in the transport of cystine into human endothelial cells: relationship to intracellular glutathione. *Biochimica et Biophysica Acta* **1222**, 375–382.

Dunham HJ, Williams C, Edens FW. 1993. Lactobacillus reuteri immunomodulation of gamma-glutamyl transpeptidase through Caco-2 cell monolayers. *Biochemical and Biophysical Research Communications* **178**, 1028–1035.

Eckmann L, Kagnoff MF. 2001. Cytokines in host defense against Salmonella. *Microbes and Infection* **3**, 1191–1200.

Ehrhardt RO, Strober W, Harriman GR. 1992. Effect of transforming growth factor (TGF)-beta 1 on IgA isotype

expression. TGF-beta 1 induces a small increase in sIgA+ B cells regardless of the method of B cell activation. *Journal of Immunology* **148**, 3830–3836.
Fioramonti J, Theodorou V, Bueno L. 2003. Probiotics: What are they? What are their effects on gut physiology? *Best Practice & Research in Clinical Gastroenterology* **17**, 711–724.
Gedek BR. 1999. Adherence of *Escherichia coli* serogroup O157 and the *Salmonella typhimurium* mutant DT 104 to the surface of *Saccharomyces boulardii*. *Mycoses* **42**, 261–264.
Gitter AH, Bendfeldt K, Schulzke JD, Fromm M. 2000. Leaks in the epithelial barrier caused by spontaneous and TNF-α induced single cell apoptosis. *FASEB Journal* **14**, 1749–1753.
Groux H, Powrie F. 1999. Regulatory T cells and inflammatory bowel disease. *Immunology* **20**, 442–445.
Haruki K, Tohno M, Shimosato T, Saito T. 2008. Development of molecular immunoassay system for probiotics via toll-like receptors based on food immunology. *Animal Science Journal* **79**, 11–21.
Huang Y, Li YL, Huang Q, Cui ZW, Yu DY, Rajput IR, *et al.* 2011. Effect of orally administered Enterococcus faecium EF1 on intestinal cytokines and chemokines production of suckling piglets. *Pakistan Veterinary Journal* **32**, 81–84.
Ichikawa H, Kuroiwa T, Inagaki A. 1999. Probiotic bacteria stimulate gut epithelial cell proliferation in rat. *Digestive Diseases and Sciences* **44**, 2119–2123.
Iji PA, Tivey DR. 1998. Natural and synthetic oligosaccarides in broiler chicken diets. *Word's Poultry Science Journal* **54**, 129–143.
Jeon SG, Kayama H, Ueda Y, Takahashi T, Asahara T. 2012. Probiotic *bifidobacterium breve* induces IL-10-Producing Tr1 cells in the colon. *PLoS Pathogens* **8**, e1002714.
Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisén J. 1999. Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* **96**, 25–34.
Kayamuro H, Abe Y, Yoshioka Y, Katayama K, Nomura T, Yoshida T, *et al.* 2009. The use of a mutant TNF-a as a vaccine adjuvant for the induction of mucosal immune responses. *Biomaterials* **30**, 5869–5876.
Khan MZ, Hashimoto Y, Iwami Y. 1997. Postnatal development of B lymphocytes and immunoglobulin-contaning plasma cells in the chicken oviduct: studies on cellular distribution and influence of sex hormones. *Veterinary Immunology and Immunopathology* **56**, 329–338.
Kim PH, Kagnoff MF. 1990. Transforming growth factor-beta 1 is a costimulator for IgA production. *Journal of Immunology* **144**, 3411–3416.
Lammers KM, Brigidi P, Vitali B, Gionchetti P, Rizzello F, Cara-Melli E, *et al.* 2003. Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells. *FEMS Immunology and Medical Microbiology* **38**, 165–172.
Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* **288**, 2222–2226.
Mestecky Y, Mcghee JR. 1992. Prospect for human mucosal vaccines. *Advance in Experimental Medicine and Biology* **327**, 13–23.
Potten CS. 1998. Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *pylori*gamma-glutamyltranspeptidase for the colonization of the gastric mucosa of mice. *Molecular Microbiology* **31**, 1359–1372.
Rajput IR, Li LY, Xin X, Huang Y, Cui ZW, Yu DY, *et al.* 2013a. Supplementation of *Saccharomyces boulardii* and *Bacillus subtilis* B10 to increase digestive enzymes activity, antioxidation functioning and blood homeostasis in broiler. *International Journal of Agriculture and Biology* **15**, 231–237.
Rajput IR, Li LY, Xin X, Wu BB, Juan ZL, Cui ZW, *et al.* 2013b. Effect of *Saccharomyces boulardii* and *Bacillus subtilis* B10 on intestinal ultrastructure modulation and mucosal immunity development mechanism in broiler chickens. *Poultry Science* **92**, 956–965.
Rajput IR, Li WF, Li YL, Lei J, Wang MQ. 2013c. Application of probiotic *(Bacillus subtilis)* to improve immunity, antioxidation, activity of digestive enzymes and haematological profile of shaoxing duck. *Pakistan Veterinary Journal* **33**, 69–72.
Rajput IR, Li WF. 2012. Potential role of probiotics in mechanism of intestinal immunity. *Pakistan Veterinary Journal* **32**, 303–308.
Sanders ME. 1993. Summary of conclusions from a panel of experts on health attributes of lactic cultures: Significance to fluid milk products containing cultures. *Journal of Dairy Science* **76**, 1819–1828.
Sherman PM, Johnson-Henry KC, Yeung HP, Ngo PSC, Goulet J, Tompins TA. 2005. Probiotics reduce enterohemorrhagic *Escherichia coli* O157:H7- and enteropathogenic *E. coli* O127: H6-induced changes in polarized T84 epithelial cell monolayers by reducing bacterial adhesion and cytoskeletal rearrangements. *Infection and Immunity* **73**, 5183–5188.
Smith TK, Gibson CL, Howlin BJ, Pratt JM. 1991. Active transport of amino acids by stressor-associated diseases in newly hatched chickens and turkeys. *Poultry Science* **72**, 100–103.
Sougioultzis S, Simeonidis S, Bhaskar KR, Chen X, Anton PM, Keates S, *et al.* 2006. *Saccharomyces boulardii* produces a soluble anti-inflammatory factor that inhibits NF-$_k$B-mediated IL-8 gene expression. *Biochemical and Biophysical Research Communications* **343**, 69–76.
Szajewska H, Skorka A, Dylag M. 2007. Metaanalysis: *Saccharomyces boulardii* for treating acute diarrhoea in children. *Alimentary Pharmacology & Therapeutics* **25**, 257–264.
Trevejo JM, Marino MW, Philpott N, Josien R, Richards EC, Elkon KB, *et al.* 2001. TNF-alpha-dependent maturation of local dendritic cells is critical for activating the adaptive immune response to virus infection. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 12162–12167.
Varcoe JJ, Krejcarek G, Busta F, Brady L. 2003. Prophylactic feeding of *Lactobacillus acidophilus* NCFM to mice attenuates overt colonic hyperplasia. *Journal of Food Protection* **66**, 457–465.
Wageha A, Ghareeb K, Böhm J. 2008. Intestinal Structure and Function of Broiler Chickens on Diets Supplemented with a Synbiotic Containing Enterococcus faecium and Oligosaccharides. *International Journal of Molecular Sciences* **9**, 2205–2216.
Yan SP, Zhang QY, Tang ZC, Su WA, Sun WN. 2006. Comparative proteomic analysis provides new insights into chilling stress responses in rice. *Molecular and Cellular Proteomics* **5**, 484–496.
Zhang X, Zhang X, Yang Q. 2007. Effect of compound mucosal immune adjuvant on mucosal and systemic immune responses in chicken orally vaccinated with attenuated Newcastle-disease vaccine. *Vaccine* **25**, 3254–3262.

