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Original Research Article

Immunohistochemical and Histomorphometric Related Small Intestine Changes Associated With Sodium Butyrate Supplement in Chickens

Key words

IL-22;
TLR8;
intestinal villi;
sodium butyrate; goblet cells;
lymphoid nodules

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Abstract: This study evaluated the effects of sodium butyrate (SB) supplementation on small intestine development in broiler chickens. Periodic acid-Schiff (PAS) and immunohistochemistry were used to undertake histological examinations of the duodenum, jejunum, and ileum. Duodenum, jejunum, and ileum histomorphometric data (villus length, crypt depth, goblet cell count), and interleukin-22 (IL-22) and toll-like receptor 8 (TLR8) immuno-stained area tissue coverage were quantified in control and SB supplemented groups. The histological changes in the SB supplemented group compared to the control group were as follows: There were increased villi lengths, widths, and crypt depths in the small intestine (duodenum, jejunum, and ileum). Increased numbers of goblet cells were observed, especially in the ileum. In addition, the lymphoid tissue within the small intestine was significantly larger (cross-section area=SB 34.8±0.5m2 vs control 13.2±0.5m2) and presented with more lymphoid nodules and more diffuse lymphoid tissue in the tunica submucosa, in the SB supplemented group compared to controls. Chickens do not have lymph nodes, therefore the mucosal-associated lymphoid tissue plays a major immunological role. Significant immunohistochemistry expression of IL-22 and TLR8 proteins were observed in the intestinal epithelial layer of the small intestine, which may play a role in protecting against many pathogens and gastrointestinal cancers.

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Introduction

Broilers are meat-producing chickens and are popular worldwide for their growth efficiency, and therefore low production cost. They grow in six weeks from an average hatching weight of around 40 g to a weight of around 2.4 kg. Conventionally farmed broilers are typically slaughtered from 42 days whereas slower growing or organic chickens are culled at 8-12 weeks (1). Broiler breeders have also concentrated on producing rapidly growing broilers in order to maintain gut health in antibiotic-free rearing programs (2). Intestinal health is critical for maximizing feed efficiency and growth rates (3, 4). Previous studies have already found a link between the development of intestinal anatomical characteristics and animal weight gains and feed conversion ratios (5).

The chicken's small intestine has three compartments (duodenum, jejunum, and ileum), each with a tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa (6). Previous histological studies have shown that the tunica mucosa is composed of surface epithelium

and lamina propria and may lack a well-developed lamina muscularis mucosae (7). The height of the villi gradually decreases from the duodenum, jejunum then ileum, and microvilli are present on the apical edge of columnar cells (8, 9). The crypts, containing intestinal stem cells and Paneth cells, have a basic tubular structure with a bulging base that opens into the lumen between the villi's bases, the interiors of the villi have multiple nodules. Previous studies have indicated that longer villi and deeper crypts increase tissue regeneration and nutrient absorption (8-10). The lamina propria consists of loose connective tissue and contains blood capillaries, a few smooth muscle cells, and lymph nodules. The tunica muscularis in chickens is divided into three layers of smooth muscle cells: the inner longitudinal, middle circular, and outer longitudinal layer. The thickness of the tunica muscularis increases as you navigate from the duodenum through to the ileum. The tunica serosa contains mostly loose connective tissue, blood vessels, and mesothelial cells (11-13).

Butyrate (a straight-chain alkyl carboxylic acid also called butyric acid and butanoic acid) and other short-chain fatty acids are widely used in animal nutrition because they assist weight gain and have a variety of other beneficial digestive tract effects (14). Sodium salt from butyric acid is commonly called sodium butyrate (SB). SB has been linked to protective effects against Salmonella enteritidis in birds (15) and butyrate has been shown to aid in the development of the intestinal epithelium (16).

Previous studies (16-18) have indicated links between enhanced immunity and SB supplementation, therefore protein expression of interleukin-22 (IL-22) and toll-like receptor 8 (TLR8) were investigated in the present study. IL-22, a cytokine involved in many processes ranging from innate immune responses to tissue regeneration, helps to regenerate many organs, including the intestine, thymus, liver, lung, and pancreas (19, 20). Agonists to TLR8, an endosomal receptor that recognizes single stranded RNA, have been used in clinical trials as immune enhancers in combination therapy for a variety of cancers (21). Despite studies reporting marked enhancements in chicken growth performance due to the use of food supplemented with sodium butyrate (22-24), the major histological changes within the small intestine have not been elucidated and requires further study.

The present study outlines the histological changes in chickens supplemented with SB relating to small intestine architecture and goblet cell count, in addition to showing the intestinal immunohistochemistry expression of IL-22 and TLR8.

Material and methods

Animal and experimental design

The study was conducted with ethical permission from the Faculty of Veterinary Medicine, Alexandria University and approved by Institutional Animal Care and Use Committee (ALEXU-IACUC; Approval code: 013/2022/12/12/189). A total of 60 one-day-old male Cobb 500 broilers were obtained from the Integrated Management and Hatching Laboratory (Desert Road Facility, Alexandria governorate). A completely randomized design was used to randomly allocate the chickens into one of two groups: the control group (n=30) and the SB supplemented group (n=30). Each group was represented by two replicates with 15 chicks per replicate, and each group of n=15 was housed in separate, controlled environment pens in accordance with Directive 2010/63/EU, separated by wooden chipboard. Control chickens received water free from sodium butyrate and SB supplemented received water containing 0.98 mg sodium butyrate (West Bengal Chemical Industries Ltd., India) per 1 ml of drinking water from days 1-28 (based on a previous study (25)).

The chicks were housed on litter (mainly wood chippings) on the floor at a depth of 5 cm and brooded at 32 °C for the first week, which was reduced by 3 °C weekly, then maintained at 20 °C by week four. Relative humidity was maintained at between 65% and 75%. Following standard healthcare regimes, the chicks were vaccinated against Newcastle disease and infectious bronchitis at the 8th and 18th days (using the Hitchner, IB, and HIPRAVIAR Clon live vaccine CL/79 clon), in their drinking water. They were also vaccinated against infectious bursal disease (IBD) on the 14th day using an intermediate strain in drinking water (26). Both groups received minerals and vitamins in their *ad lib* feed (full ingredients shown Table 1) as per Nutrient Requirements of Poultry guidelines (27) with a starter diet provided on days 0 to 14, then a grower diet containing from day 15 to until the end. Fosfomycin antibiotics were added to their water as standard animal care (therefore bacterial load was not investigated in the present study). The chicks had daily health checks and underwent cervical dislocation and decapitation on day 30.

Histological and immunohistochemical (IHC) examination of the small intestine

Fresh samples from the duodenum, jejunum, and ileum were collected from n=5 per replicate control and n=5 per replicate SB supplemented groups for histological examination. The specimens (duodenum, jejunum, and ileum) were fixed in 10% phosphate-buffered formaldehyde, processed into paraffin blocks and 5µm sections cut then stainedLayton, Bancroft and Suvarna (28). The periodic acid-Schiff technique (PAS) was used to identify goblet cells using a previously published protocol (29). In brief this consisted of 0.5 % periodic acid for 10 min, Schiff's Reagent

Table 1: Ingredients and nutrient composition (% dry mass) in broiler starter and grower food

Ingredient	Starter (%) days 1-14	Grower (%) days 15-30
Yellow corn	53.5	66.5
Soybean meal (48% starter mix)	34.38	24
Corn gluten	5.82	3.6
Salt	0.3	0.3
Dicalcium phosphate[A]	2.7	2.48
Vitamin premix[B]	0.3	0.3
Corn oil	3.00	2.82
Total	100%	100%
Calculated values		
Metabolizable Energy (ME) (kcal/kg)	2976.83	3096.31
Crude protein	22.97	18.07
Calcium	1.08	0.9
Available phosphorus	0.52	0.45
Methionine[C]	0.52	0.51
Lysine[D]	1.29	1.13

A) Dicalcium phosphate, 18% granular phosphate, and 23% calcium. B) Supplied per kg of diet: vitamin A 12,000 IU, vitamin D3 3,000 IU, vitamin E 40mg, vitamin K3 3mg, vitamin B1 2mg, vitamin B2 6mg, vitamin B6 5 mg, vitamin B12 0.02mg, niacin 45mg, biotin 0.075mg, folic acid 2mg, pantothenic acid 12mg, manganese 100mg, zinc 600mg, iron 30mg, copper 10mg, iodine 1mg, selenium 0.2mg, cobalt 0.1mg. C) DL-Methionine, Met AMINO® (DL-2- - amino-Y-methyl-oily acid)amino-4-(methyl-thio)-butane acid, DL-methionine, by Feed Grade 99% (EU). D) L-Lysine HCL 99% (Feed Grade) L-Lysine: 78.0% Min (Indonesia)

Table 2: List of antibodies, their sources, working dilutions and antigen retrieval

Antibody	Catalogue number and Source	Dilution	Antigen retrieval
Rabbit polyclonal anti-IL-22	ab18564, Abcam, UK	1:100	Tris-HCl, 110°C 15 min microwave
Rabbit polyclonal anti-TLR-8	ab24185, Abcam, UK	1:1000	10 mM citrate buffer (pH 6.0), 105°C 20 min microwave
Biotin-conjugated goat anti-rabbit IgG antiserum (secondary)	Histofine kit 424032, Nichirei Biosciences Inc. Japan		

Table 3: Histomorphometric changes in the small intestine following administration of dietary sodium butyrate

	Villus length (µm) ± SEM		Crypt depth (µm) ± SEM		Goblet cell count/10,000 µm² ± SEM	
	SB group	Control group	SB group	Control group	SB group	Control group
Duodenum	374.67±0.83[a]	360.00±0.94[b]	244.000±0.54[a]	173.000±0.47[b]	1306.66±15.15[a]	294.67±3.18[b]
Jejunum	574.67±0.83[a]	400.67±1.44[b]	163.666±2.16	150.666±3.66	702.66±0.57[a]	531.67±0.98[b]
Ileum	1000.00±2.72[a]	421.33±5.19[b]	623.666±0.57[a]	335.000±0.47[b]	924.00±0.54[a]	479.67±8.04[b]

a and b = values differed significantly between the control and sodium butyrate (SB) groups, where $P < 0.05$ using ANOVA with post-hoc testing. SEM = standard error of the mean. N=10 chickens per group (control and SB).

for 20 min at 65 °C, and hematoxylin for visualization of nuclei. The goblet cells were counted using image J/Fiji in ten different fields of view for each intestinal region from each specimen at x400 magnification.

The immunohistochemical technique used for the intestinal sections was as previously published (30). The antibodies used for immunohistochemistry, and their sources and working dilutions, in addition to antigen retrieval methods, are listed in Table 2. In brief 0.5% TritonX-100 was applied for 20 min, followed by antigen retrieval (Table 2), washed in distilled water, followed by 0.3% H_2O_2/methanol for 20 min to deactivate endogenous peroxidase. Then 5% bovine serum albumin in PBS was applied followed by primary antibody incubation (Table 2).

Negative control sections were maintained in the 5% bovine serum. Followed a wash in PBS the sections were incubated for 30 min in, biotin-conjugated goat anti-rabbit IgG antiserum (Histofine kit, Cat: 424032, lot: H1401; Nichirei, Tokyo, Japan), then washed in PBS, then incubated in streptavidin–peroxidase conjugate for 30 min at room temperature. Peroxidase/3,3'-Diaminobenzidine (DAB; ChemMate Detection Kit; Cat: K5007, lot: 00054660A, Dako, Carpinteria, CA, USA) was used to visualise the conjugate and Mayer's hematoxylin was used to counterstain.

Imaging, measurements, and statistical analysis

Micrographs of the stained sections (10 systematically randomly chosen fields from every specimen for the duodenum, jejunum, and ileum respectively) were taken with a digital camera (Leica EC3, Leica, Germany) connected to a light microscope (DM500, Leica, Germany). The histomorphometric data, consisting of villi lengths, crypt depths, goblet cell counts, from within the duodenum, jejunum, and ileum, and the Peyer's patch areas and numbers in cross sections of the ileum, in both the control and SB supplemented groups were measured with the aid of image J software (National Institutes of Health, USA; Table 3). Goblet cell count was calculated on PAS sections at X400 magnification using ten different fields (each measuring 10,000 μm^2) per chick per tissue type. For quantification of immunostaining intensities, Image J software was used as described previously (31). In brief, the mean density of staining was determined in the 10 systematically randomly chosen fields from each section from the five birds in each group, as previously published by Vis and coauthors (32). This was presented as a percentage of positive immune-stained area (brown colour for DAB) for both IL-22 and TLR 8 from small intestine tissue in the control and SB supplemented groups.

Data measured as lengths and areas were statistically analyzed with one-way analysis of variance (ANOVA) and Bonferroni post-hoc testing (SPSS version 26, IBM). This enabled concomitant comparisons between the two groups (Control vs SB) and the three different tissue types (duodenum, jejunum, and ileum). Immunostaining was calculated as percentage coverage. Data are expressed as mean ± standard error of the mean (SEM), P values <0.05 were considered significant. Analyses of the significant main effects of experimental treatments were performed using multiple range comparisons with Duncan's multiple range test.

Results

Histological examination of the small intestine

The small intestines from the 60 day old chicks from both the control and SB supplemented groups had a small intestine structures consisting of four layers, the tunica mucosa, tunica submucosa (Fig.1a,e/SU), tunica muscularis (Fig.1a,e/TM), and tunica serosa (Fig.1a,e/TS). The duodenum in these chicks lacked the Bruner's gland (Fig.1a/IG) and had finger-like projecting intestinal villi (Fig.1a,b). The jejunum contained tube-like villi (Fig.1c,d), and the ileum had leaf-like villi with broad bases and pointed ends (Fig.1e,f).

Histomorphometry changes were observed in the SB groups compared to the control chicks at day 60. The length of villi (Fig.1a-f/VL, Table 3) increased in the SB group duodenum, jejunum, and ileum compared to controls ($P < 0.05$). Crypt depth (Figs.1a-f/CD; 2a-b, Table 3), increased in the SB group duodenum and ileum compared to controls ($P < 0.05$) but not the jejunum ($P > 0.05$). The goblet cells had a characteristic magenta-red color and had a flask shape between epithelial cells of the intestinal villi (Figs. 2 and 3). The number of goblet cells significantly increased in the duodenum (control 360.00±0.94, SB 374.67±0.83) jejunum (control 400.67±1.44, SB 574.67±0.83) and ileum (control 421.33±5.19, SB 1000.00±2.72) with the administration of sodium butyrate in the supplemented group (Figs.2c and 3b/arrowheads) in comparison with the control group (Figs.2c and 3a/arrowheads; $P < 0.05$; Table 3).

The surface area of each of the lymphoid nodules, known as Peyer's patches within the ileum, increased in the SB supplemented group to 34.3±0.5 μm^2 in comparison with the control group at 13.3±0.3 μm^2 (Figs.1e&f arrows and 4a–b/LN; $P < 0.05$). Concomitantly, the number of Peyer's patches was higher in the SB group compared to controls (19±0.45 in SB versus 10.8±0.49 in controls; Fig.4b/LN; $P < 0.05$). The combined surface area with number of nodules resulted in an increased nodule area in the SB group (651.68 μm^2 in controls versus 143.56 μm^2 in SB supplemented per cross section of the ileum; $P < 0.05$). Microscopic qualitative observation of the sections indicated that the SB supplemented group showed a few regressed intestinal glands (Fig.4b/arrows), and instead of diffuse glands, aggregated lymphoid tissue was observed (Fig.4b/DL). Qualitative analysis also showed that in the SB supplemented group ileums, there were diffuse

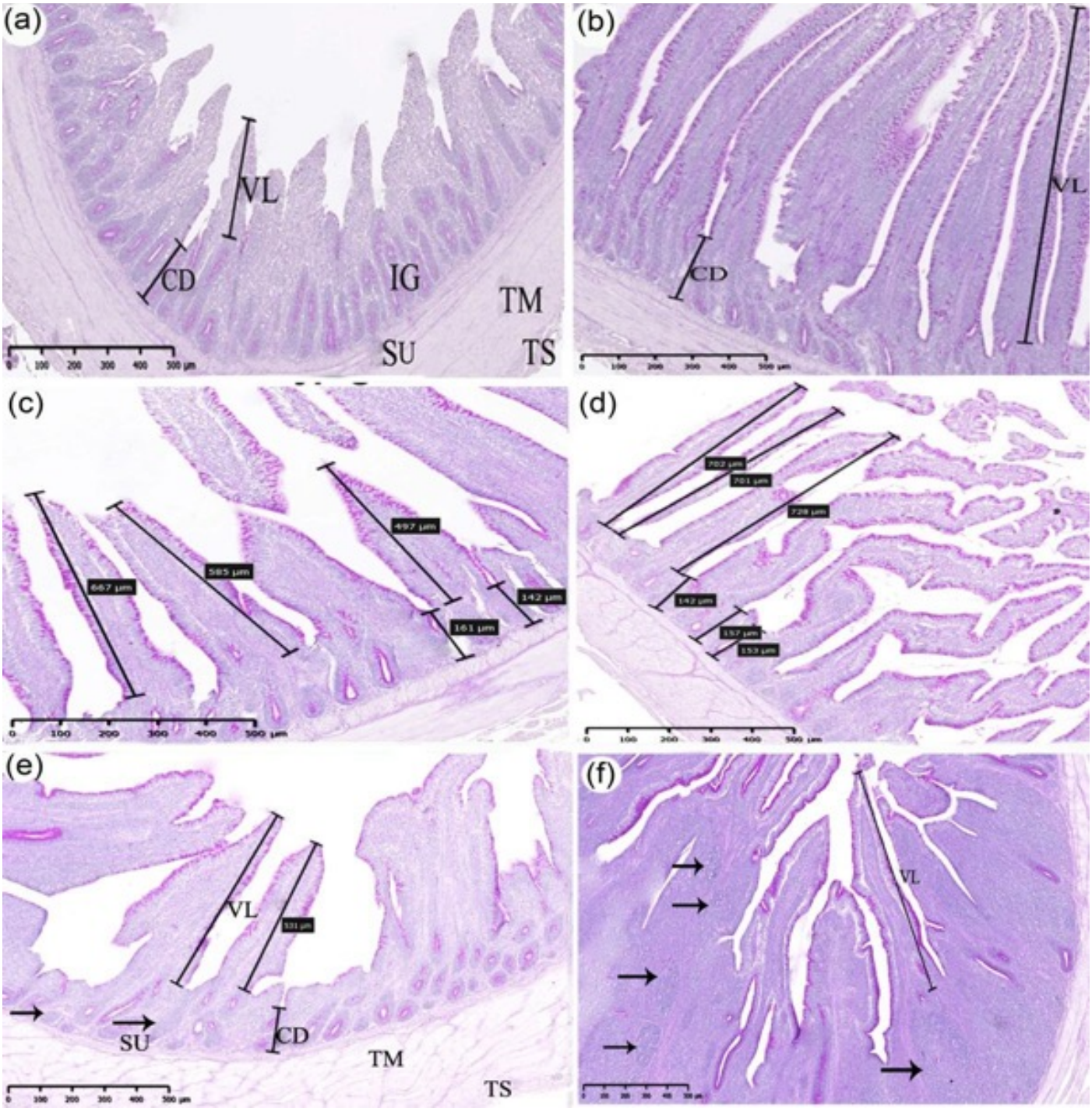

Figure 1: Photomicrographs of the duodenum, jejunum and ileum in control and SB supplemented chickens

a) Control group duodenum showing villi (finger-like shape). b) SB supplemented duodenum showing increased villus length and crypt depth. c) Control group jejunum showing villi length and crypt depth. d) SB supplemented group jejunum with increased villi length and crypt depth. e) Control group ileum indicating villus length and crypt depth, with associated tunica submucosa, tunica muscularis, tunica serosa, and fewer numbers of lymphoid nodules (arrow). f) SB supplemented group ileum with increased villus length, increased numbers of lymphoid nodules which were larger in size (arrow). Villus length (VL). Crypt depth (CD). Intestinal gland (IG). Tunica submucosa (SU). Tunica muscularis (TM). Tunica serosa (TS). Stain = PAS, Magnification = 40X, scale bars represent 500 µm

lymphocytes present (Fig.4c/I), penetration of lymphocytes into the diffuse lymphoid tissue had occurred and the cells were located next to enterocytes (Fig.4c/arrows), desquamated epithelium were observed (Fig.4c/DS), and mucous secretion was observed in the lumen near the tip of the villi in the ileum (magenta-red color in Fig.4c/MU and arrowhead). In addition, a lymph vessel lined with simple squamous endothelial cells each containing a flat and elongated nucleus (Fig.4d/arrowheads) was observed, the lymph vessel was filled with lymphocytes (Fig.4d/L). Cells

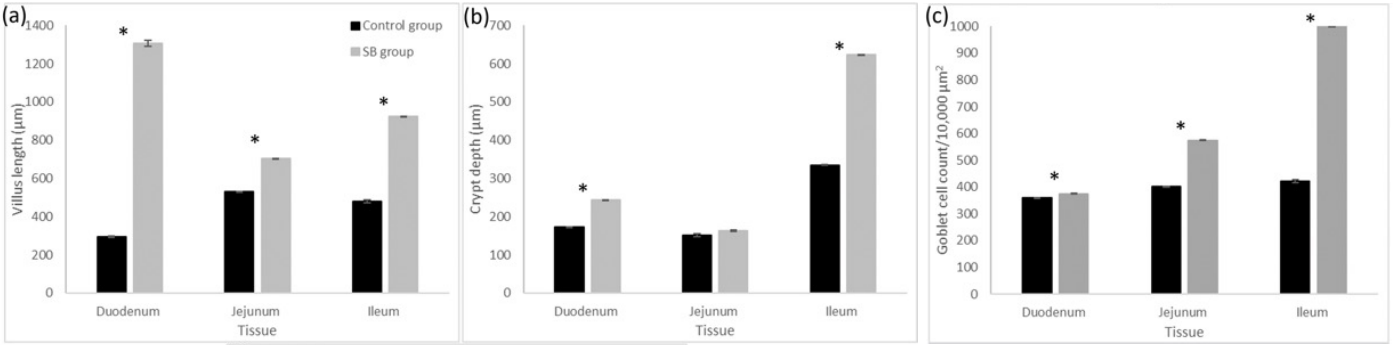

Figure 2: Villus length, crypt depth, and goblet cell number measurements within the small intestine. a) Villus length, b) crypt depth and c) goblet cell quantification in the duodenum, jejunum, and ileum. Calculated on PAS sections at X400 magnification using ten different fields (each measuring 10,000 µm²) per chick per tissue type (duodenum, jejunum, and ileum). Values represent mean ± SEM. * indicates significant differences P < 0.05 between control and SB supplemented groups

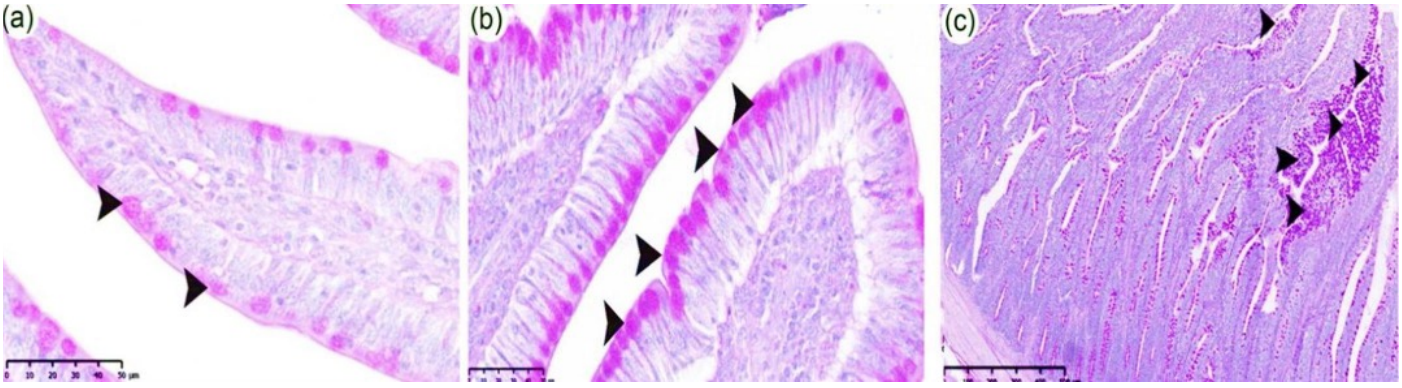

Figure 3: Goblet cells within the ileum and jejunum. (a) Control group ileum had a low density of goblet cells (arrowheads). (b) SB supplemented group ileum exhibiting increased numbers of goblet cells, with a higher density in a typical cup shape-like structure (arrowheads). (c) SB supplemented group jejunum showing hyperplasia of goblet cells within the jejunal villi (arrowheads). Stain = PAS. a+b) Magnification = X400, scale bars represent 50µm, c) Magnification = X40, scale bars represent 500 µm

that looked like macrophages with kidney shaped nuclei (Fig.4d/arrow) and diffuse lymphoid tissue were located under the lamina epithelial of the dome structure of Peyer's patches in the lamina propria, known as loose connective tissue (Fig.4d/DL), were also observed. Following SB supplementation for 60 days, a high number of reticular fibers (Fig.4e/arrows) were noted, potentially acting as a scaffold for diffuse lymphoid tissues (Fig.4e/DL) in the lamina propria of the ileum (Fig.4e).

Immunohistochemistry analysis and immune response

Expression of IL-22 in the small intestine tissue (duodenum, jejunum, and ileum) was observed in both the SB supplementation group and the control group (Fig.5, Supp Table 1). The duodenum, jejunum, and ileum intestinal tissue in the control group revealed weak expression of IL-22 (Fig.5a,c,e). Conversely, SB supplemented chickens exhibited noticeable darker IL-22 staining in the duodenum, jejunum, and ileum, within the epithelium lining the villi (Figs.5b,d,f/arrows and 6a) and crypts (Fig.5b,d,f/arrowheads). TLR8 protein expression was present in the epithelium lining of the small intestine (duodenum, jejunum, and ileum) tissue. As observed with IL-22, the control group revealed weak expression of TLR8 (Fig.7a,c,e/arrows), whereas the SB supplemented group exhibited notably darker TLR8 expression in the epithelium lining of

Supplementary Table 1: Quantification of interleukin-22 (IL-22) and Toll-like receptor 8 (TLR8) expression

	SB supplemented (%)	Control (%)
IL-22 - Duodenum	16.23±0.12	1.33±0.03
- Jejunum	13.43±0.03	1.87±0.03
- Ileum	16.07±0.07	2.07±0.07
TLR8 - Duodenum	23.68±0.33	5.40±0.06
- Jejunum	22.16±0.09	4.30±0.11
- Ileum	24.83±0.44	5.37±0.09

Immunohistochemical positive staining was measured as total area (expressed as a percentage of tissue) across ten different fields per tissue sections. Values represent mean±SEM.

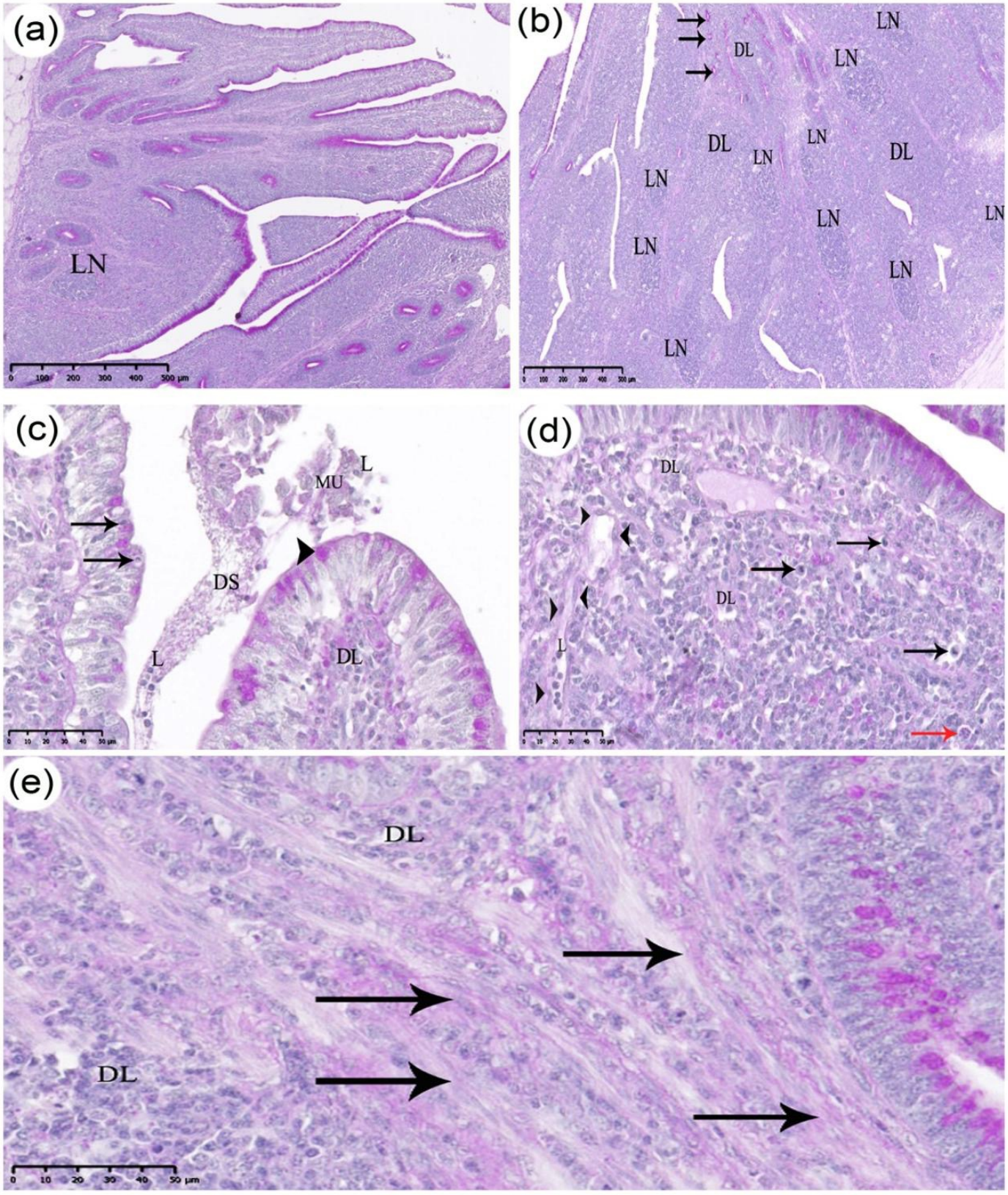

Figure 4: Histological observations in the ileum tissue. a) The control group ileum exhibited very few lymphoid nodules (LN). b) In the SB supplemented group the ileum contained a few regressed intestinal glands (arrows), as most had been replaced by diffuse lymphoid tissue (DL) and a high number of Peyer's patches (LN). c) In the SB supplemented group ileum there was penetration of lymphocytes into the diffuse lymphoid tissue (arrows), desquamated epithelium (DS), lymphocytes (L), magenta-red color mucous secretion (MU) near to tip of the villi of the ileum (arrowhead), and diffuse lymphoid tissue in lamina propria (DL). d) In the SB supplemented groups ileum lymph-vessel (arrowheads), lymphocytes were present inside the lymph vessel (L), beneath the dome structure, and between diffuse lymphocytes in the lamina propria (DL), and macrophages with kidney shape nuclei (arrows). e) The SB supplemented ileum also exhibited reticular fibers-stained magenta-red in color (arrows), with diffuse lymphoid tissue in the lamina propria (DL). Staining = PAS. a+b) Magnification = X40, scale bars represent 500 µm. c-e) Magnification = X400, scale bars represent 50 µm

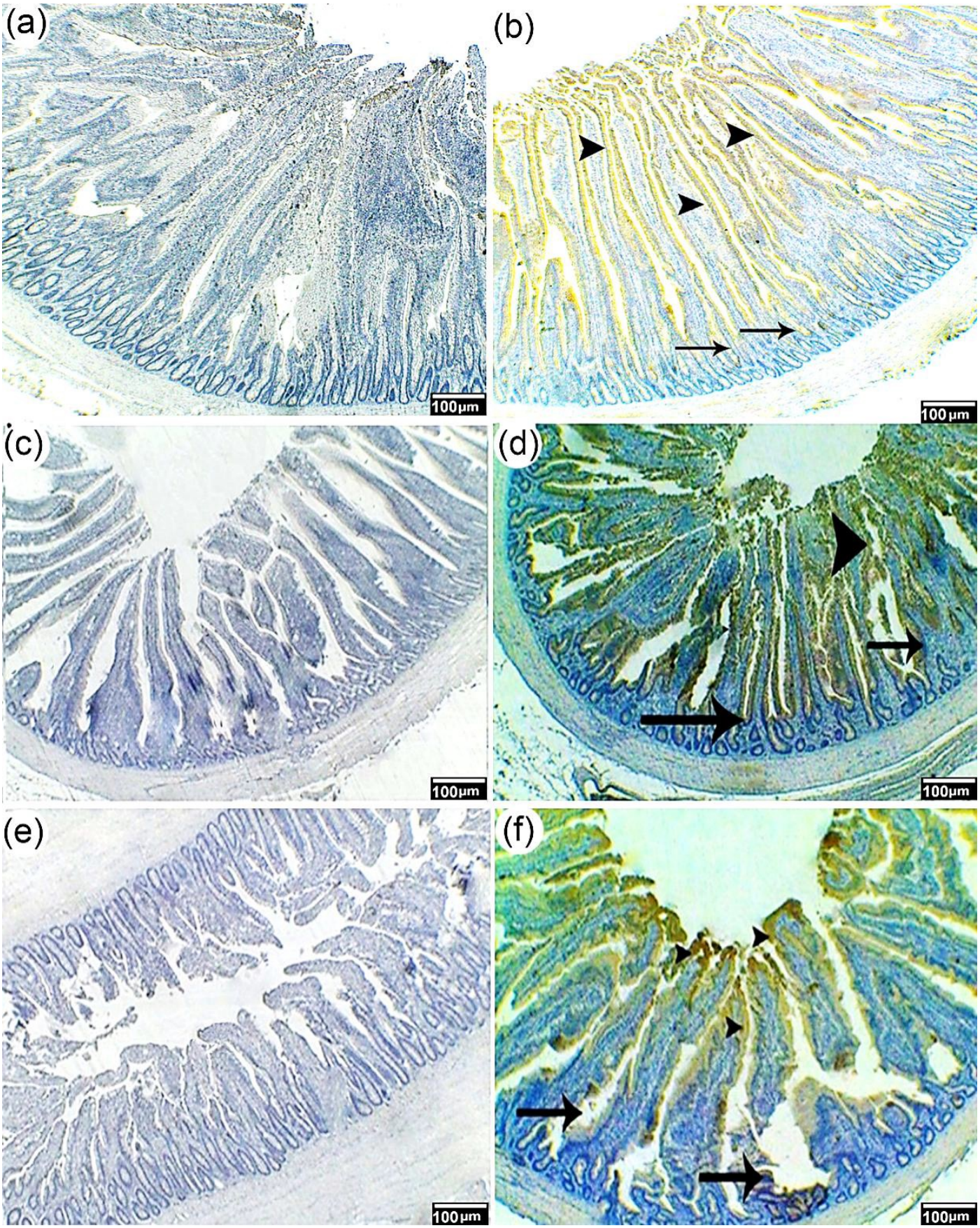

Figure 5: Mucosal cell immunohistochemical staining for interleukin-22 (IL-22) in the small intestinal (duodenum, jejunum, and ileum) in the epithelium lining the villi (arrows) and crypts (arrowheads). a) Control group duodenum, b) SB supplemented group duodenum, c) Control group jejunum, d) SB supplemented group jejunum, e) Control group ileum and f) SB supplemented group ileum. Note the DAB brown color denoting IL-22 expression in the SB supplemented intestinal tissue. Magnification = X40, scale bar represents 100 µm

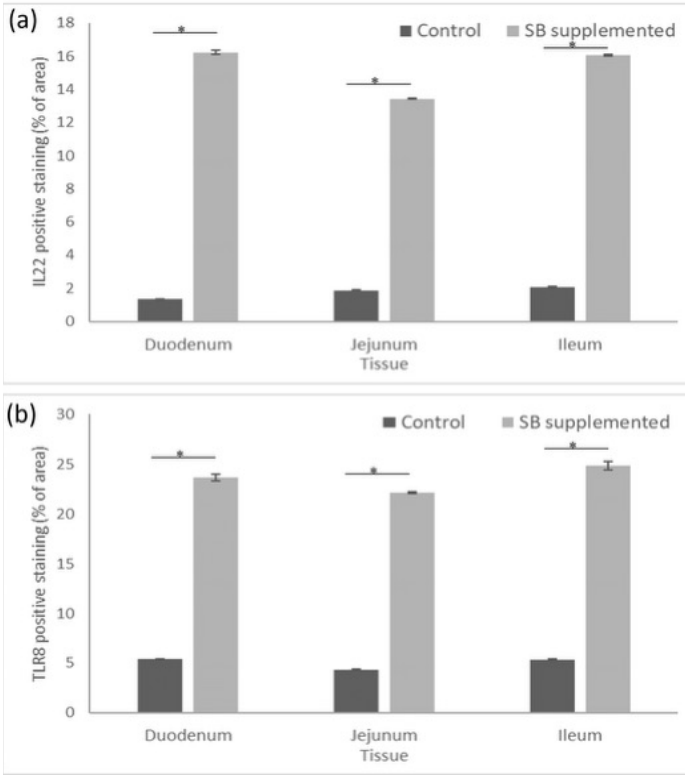

Figure 6: Quantification of a) interleukin-22 (IL-22) and b) Toll-like receptor 8 (TLR8) expression. Immunohistochemical positive staining was measured as total area (expressed as a percentage of tissue) across ten different fields per tissue section. Values represent mean ± SEM. * indicates significant differences between control and SB supplemented groups ($P < 0.05$)

the duodenum, jejunum, and ileum epithelium (Fig.7b,d,f/ arrows, dark brown immune-positive staining).

Concomitant with the qualitative changes described above, there was a quantitative increase in the mean area (expressed as a percentage of total tissue) of IL-22 positive immuno-stained tissue in the SB supplemented group compared to the control group. The percentage of immunostained areas were control 1.33±0.03 vs SB 16.23±0.12% in the duodenum, control 1.87±0.03 vs SB 13.43±0.03% in the jejunum, and control 2.07±0.07 vs SB 16.07±0.07 in the ileum. Quantification of TLR8 protein expression also showed more tissue area was immunostained in the SB supplement intestinal tissues compared to control tissues (duodenum control 5.40±0.06 vs SB 23.68±0.33%, jejunum control 4.30±0.11 vs SB 22.16±0.09%, ileum control 5.37±0.09 vs SB 24.83±0.44%.

Discussion

In this study, the administration of sodium butyrate significantly increased duodenal, jejunal, and ileum villi lengths and crypt depths, similar to previous reports in broiler chickens using coated SB (33, 34). Longer villi are thought to support larger surface areas, enabling higher absorption capacities and healthier intestinal development, resulting in the gut's optimal state (35-37). Butyrate is the active ingredient within sodium butyrate which was absorbed by enterocytes as a source of energy, it has therefore been implemented to support faster intestinal development and function as well as facilitating improved overall health (38, 39). Furthermore, in the present study microscopic analysis of the villi revealed that SB supplements increased the number of goblet cells by 4% in the duodenum, 43% in the jejunum and 137% in the ileum compared to controls. Microscopic observations also indicated the SB supplemented group chicks exhibited potential hyperplasia of the goblet cells within the jejunal villi (Fig.3c/arrowheads, qualitative observation). Goblet cells are responsible for the production and stimulation of mucus (40) and the mucus layer is often considered the first line of defense in the intestinal mucosa. The mucins were controlled by changing the number of goblet cells or mucin gene expression, especially MUC2 (41). Therefore, it is of interest that SB supplementation increased the number of goblet cells, therefore potentially impacting mucus production and immunity.

Chicks do not have lymph nodes, and the present study confirmed the lack of lymph nodes, therefore it has been asserted that the mucosal-associated lymphoid tissue has a major immunological role. Eren and coauthors (42) explained the role of cecal tonsils as one mucosal associated lymphoid tissue in chicken and highlighted its role in TLR2 and TLR4. An important finding of the present research related to the ileum lymphoid nodules, known as Peyer's patches, which increased in both number and size, resulting in an overall increase in tissue following SB supplementation. The surface area of each of the Peyer's patches within the ileum increased by 2.5-fold in the SB supplemented group in comparison with the control group. Concomitantly, the number of Peyer's patches was 75% higher in the SB group compared to controls, and when the surface areas and number of nodules were considered together, this resulted in a 4.5 increase in nodule area in the SB group. This finding agrees with Sikandar *et al.*, (34) who recorded that coated SB increased the size and number of lymphoid nodules in the bursa and that SB also enhanced immune system function. In the present study the lymph vessels within the ileum were lined with a single layer of endothelium and filled with lymphocytes. This finding agrees with the review written by Pepper and Skobe (43) which suggested that the lymph vessels had significant importance for the entrance of leukocytes and serving immunological functions. In the present study relatively high numbers of macrophages were observed in the lamina propria of the ileum. This finding concurs with concepts as previously reviewed (44) which explained the role of macrophages in the immunological defense in Peyer's patches of the ileum of mammals known for phagocytic activities.

Reticular fibers were evident in the Peyer's within the ileum of both control and SB supplemented, but more evident in

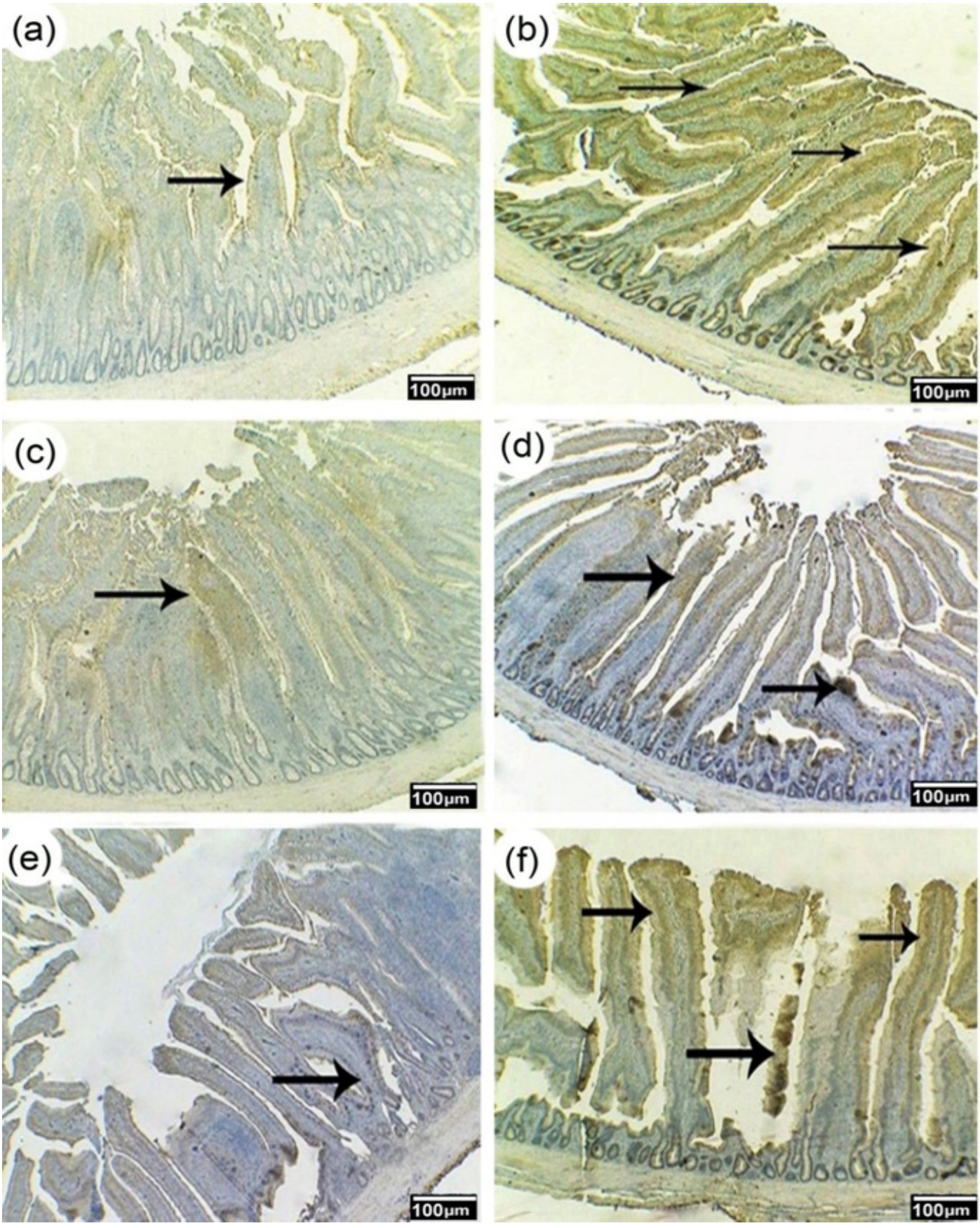

Figure 7: Immunohistochemical staining of Toll-like receptor 8 (TLR8) in the epithelium lining and intestinal mucosal cells in the duodenum, jejunum, and ileum - arrows. a) Control duodenum, b) SB supplemented duodenum, c) Control jejunum, d) SB supplemented jejunum, e) Control ileum, f) SB supplemented ileum. Note the pale brown DAB immunostaining expression of TLR8 in the control group compared to the darker dark brown in the SB supplemented group. Magnification = X40, scale bar represents 100 µm

the SB chickens. The reticular fibers were visualized as a magenta-red color following PAS application, indicating their structure rich with heparin sulfate, which belong to the glycosaminoglycans (a polysaccharide), as explained by Montes and coauthors (45). The lymphocytes in the present study, which move from the submucosa to the epithelium tissue, were found between the enterocytes in the ileum lamina epithelial layer. This finding agreed with Lundqvist *et al.*, (46) who observed a similar situation in the human small intestine. They summarized that the immunological role of intraepithelial lymphocytes, a lytic role and had potential effects as T-helper and T-cytotoxic lymphocyte functions, which had a cytolytic role in tumor cells. The intestinal epithelium also played a barrier role, that was enhanced by sodium butyrate, in a mouse model of diabetes and obesity, helping to ameliorate diabetic-endotoxemia (37).

Sodium butyrate supplementation had a positive relationship with interleukin-22 immunostaining expression in the intestinal epitheliums in the small intestines of the SB supplemented group chicks. This finding agrees with Yang and coauthors (47), who reported that butyrate enhanced IL-22 production in vivo (in mice) and played a role in the inhibition of colitis via IL-22 action. Both our present study, and the previous work in mice, have indicated that SB supplementation therefore plays a positive role in intestinal immunity. For example Keir et al., (48), reviewed the role of IL-22 in intestinal health and disease, and discussed the role of IL-22, functioning as an intestinal barrier, with roles in intestinal hemostasis, complement production, and cancer. Patnaude and colleagues (49) concluded that the mechanisms behind IL-22 in human intestinal organoids and resections, and mice, by showing enhanced expression of the stem of intestinal cells, cell division, the intestinal mucous layer, and barrier activities against many pathogens. A review by Chen and coauthors (50) discussed that butyrate, T cells, and intestinal epithelium cooperated in environmental harmony to act in an anti-apoptosis, pro-proliferation, and anti-inflammation manner. As IL-22 responses were found in both healthy and diseased states, they suggested that IL-22 had a significant role in the initiation of defense action and cell regeneration. They also suggested that further studies should occur in diseased states to help with IL-22 based therapy. However, the previous studies relating to intestinal epithelium permeability are limited, and it has been suggested that in mice IL-22 has limited action on permeability in some junctional molecules. IL-22 has also been shown to induce the action of claudin-2 regulation, as reported in many studies (51, 52). The significant increases in IL-22 expression in the present study therefore indicate potentially beneficial changes following SB supplementation in the chicken.

Sodium butyrate, a supplement, had a positive correlation with the immune expression of TLR8 in the intestinal epithelium in the developing chicks in the present study. This finding agrees those shown previously (53) in bovine mammary epithelial cells and in intestinal porcine enterocyte cultures (54). They found that the administration of sodium butyrate enhanced the expression of TLR8 and TLR2, which played a significant role in the production of antimicrobial agents to protect against many pathogens such as *Staphylococcus aureus*. SB has also been shown to alleviate the action of lipopolysaccharides, which are the main factor in intestinal injury and microflora disturbance in rats. Dou and coauthors (55) explained the mechanism of sodium butyrate action by decreasing mRNA expression of cytokines to decrease the inflammatory process, thus enhancing the microbiota's action to decrease apoptosis of the intestinal epithelium which decreased inflammation. In people, TLR8 is found intracellularly in the normal state and has a known role in innate and acquired immunity (56, 57). TLR8 has also been used to induce the activity of natural killer cells against tumors, and has been used in both tumor adjuvant and immunotherapy (58).

Conclusion

Administration of supplemental sodium butyrate (SB) via drinking water induced significant histological changes and immunological increases during intestinal development. Sodium butyrate dietary supplementation had a potentially beneficial effect on the chicken small intestine (duodenum, jejunum, and ileum) by increasing the lengths of villi, depths of crypt, number of intestinal goblet cells, the amount of diffuse lymphoid tissue, and the number and sizes of Peyer's patches. Expression levels of both IL-22 and TLR8 were also increased in the sodium butyrate supplemented group. These characteristics produced by SB supplementation may enhance the optimal intestinal microstructure and provide additional intestinal protection against many pathogens.

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Abbreviations. SB Sodium butyrate, IL-22 Interleukin-22, TLR8 Toll-like receptor 8.

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Imunohistokemične in histomorfometrične spremembe tankega črevesa, povezane z dodatkom natrijevega butirata pri piščancih

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Izvleček: Raziskava je ocenila učinke dodajanja natrijevega butirata (SB) na razvoj tankega črevesa pri piščancih brojlerjih. Za histološke preiskave dvanajstnika, teščega in vitega črevesa sta bili uporabljeni periodična kislina-Schiff (PAS) in imunohistokemija. Histomorfometrični podatki za dvanajstnik, tešče in vito črevo (dolžina resic, globina kripte, število čašastih celic) ter imunohistokemična obarvanost tkiva z interlevkinom-22 (IL-22) in Tollu podobnim receptorjem 8 (TLR8) so bili izmerjeni v kontrolni skupini in skupini, ki je prejemala dodatek SB. Histološke spremembe v skupini z dodatkom SB so bile v primerjavi s kontrolno skupino naslednje: v tankem črevesu (dvanajstniku, teščem in vitem črevu) so se povečale dolžine, širine in globine kript. Povečano je bilo število čašastih celic, zlasti v vitem črevesu. Poleg tega je bilo limfatično tkivo v tankem črevesu v skupini z dodatkom SB v primerjavi s kontrolno bistveno večje (površina prečnega prereza = SB 34,8 ± 0,5 m2, pri kontrolni skupini pa 13,2 ± 0,5 m2) in je pokazalo več limfatičnih vozličev ter bolj razpršeno limfatično tkivo v podsluznici. Piščanci nimajo bezgavk, zato ima limfatično tkivo sluznice črevesa pomembno imunološko vlogo. V epiteliju tankega črevesa smo opazili značilno imunohistokemično izražanje proteinov IL-22 in TLR8, kar lahko ima vlogo pri zaščiti pred številnimi patogeni in rakom prebavil.

Ključne besede: IL-22; TLR8; črevesne resice; natrijev butirat; čašaste celice; limfatični vozliči