Official Journal Issued by Faculty of Veterinary Medicine

**Benha Veterinary Medical Journal**

Journal homepage: https://bvmj.journals.ekb.eg/

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*Original Paper*

# Effects of Dietary Sodium butyrate on Innate Immunity and Gut Health of Broiler Chickens Challenged with *Eimeria maxima*

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**ARTICLE INFO**

**Keywords**

*Sodium butyrate*

*Eimeria maxima.*

*Inflammatory cytokines*

*Broiler*

*Received* 26/10/2022
*Accepted* 11/11/2022
*Available On-Line* 15/01/2023

**ABSTRACT**

This study was accomplished to evaluate the effect of sodium butyrate (SB) as a feed additive on the intestinal immune response against *Eimeria maxima* infection and histomorphological structure of intestine of broiler chickens. One-hundred-day-old broiler chicks were divided into 4groups, 25per each. 1st group was fed on a normal diet only. 2ndgroup was fed on a normal diet and infected with 1.0 ml of a solution containing $5.0 \times 10^3$ sporulated oocysts of *E. maxima* on the 22nd day of age. 3rd group was fed on normal diet + SB (1gm/kg feed) and infected as group two. 4th group were fed on normal diet + SB (1gm/kg feed). Five birds from each group were euthanized on the 5th,10th, and 15th days post-infection and blood and tissue samples were collected to evaluate the difference in jejunum and ileum histopathology and determination of inflammatory cytokines in serum. Results revealed significant elevation in intestinal villi length and crypt depth in treated group compared to control group. SB has significantly increased intestinal immunity against *E. maxima* infection, there were decreases in the number of coccidial stages within the mucosal lining in infected treated groups compared to infected non-treated groups, there was a decrease in enteritis feature with noticeable intestinal regeneration in group 3 compared to group 2 that showed necrotic enteritis associated with sever sloughing of the epithelial lining. SB modulated the expression of immune-related genes in terms of increased inIL-10 and decreased (IL-1β &INF-γ) gene expression.

## 1. INTRODUCTION

Gastrointestinal tract of broilers is not only an organ of feed digestion and absorption of nutrients, but also plays an important role in systemic immunity and acts as a barrier function through the mucoid layer that covers the epithelial lining of the intestine. Under the condition of intensive farming and high stocking densities broiler chickens are subjected to different stressors, mostly immunological stress (Zulkifli, Al-Aqil et al. 2009) that can affect intestinal barrier structure and functions (Jiang, Sun et al. 2009). Keeping a healthy intestine can be achieved by using feed additives especially short-chain fatty acids (SCFA) instead of using antibiotics with sub-therapeutic doses that has public health concern due to antimicrobial resistant and residues(Lesson et al.2005).

SB is one of the most important SCFA that is used as a feed additive in poultry industry. Butyrate present as sodium or calcium salt since butyric acid is volatile and has an unpleasant odour. SB is more stable and less odorous (Guilloteau et al. 2010; Jiang et al.2015).SB is the source of butyric acid (BA) which has an important role in gut development. Once SB reaches the proventriculus and gizzard it releases sodium and BA. Using a coated form of SB, control its release on the intestinal tract and allow it to reach distal part of intestinal tract. Importance of SB could be increased when it is used in coated form (Smith et al. 2012). To produce its bactericidal effect, SB is required to be in undissociated form to get access inside the bacterial cell, so it's preferable in coated form.

SB is one of the most important fatty acids that improve intestinal immunity and anti-inflammatory responses (Chang et al. 2014; Kanai et al. 2015). BA produced from SB after digestion is known for its protective and antibacterial effect, elevates the intestinal integrity, increases resistance to immunological stress and prevents *E. maxima* and *salmonella enteritidis* infections as well as protects against necrotic enteritis (Ali et al. 2014; Liu et al. 2019).SB increase villus length in the ileum of broiler (Qaisrani et al. 2015; Wu et al. 2018); therefore, when used as a feed additive in broiler cause intestinal epithelium development (Qaisrani et.al. 2015). Lesson et al. (2005) detected that after SB supplementation to broiler; there was increase in villi height and crypt depth. Sayrafi et al. (2011) found that there were significant increases in duodenal crypt depth after butyrate glycerides feeding to broiler. Butyrate seems to affect intestinal barrier by increasing mucin glycoprotein formation (Willemsen et al. 2003).

SB modifies the inflammatory responses, the mechanism of inflammation is regulated by inflammatory cytokines and pro-inflammatory genes, SB directly decreases the production of pro-inflammatory cytokines genes such as

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interleukin -1 beta(IL-1$\beta$) and interferon gamma (IFN-$\gamma$) (Galvez et al. 2003).SB increases anti-inflammatory cytokines as inter leukin -10 (IL-10) (Zou et al. 2019). Butyrate has role as a stimulator for intestinal epithelium growth, broiler performance and intestinal micro biota composition (Czerwiński et al. 2012).

Coccidiosis has a major economic effect on poultry as it reduces growth performance and decreases productivity (Zhang et al. 2013). Coccidia infection causes enteritis and tissue damage, intestinal bleeding, metabolic disturbance, and mortalities. The lesion caused by *Eimeria* infection make a disturbance in nutrient absorption that affect growth. So, in poultry farms use anticoccidial and chemoprophylaxis feed additives to avoid coccidiosis but using of this substance has public health concern.

Therefore, the current study was designed to explore effects of SB as a feed additive on the intestinal immune response, intestinal tissue architecture and expression of inflammatory cytokines against infection with sporulated oocysts of *E. maxima* in broiler chicken.

## 2. MATERIAL AND METHODS

*2.1. Experimental chicks:*

One-hundred-day-old broiler chicks Cobb breed were obtained from El-Dakhlea Poultry Company, MitGhamer city, Egypt. Chicks were kept on litter under standard environmental and hygienic conditions. The temperature was adjusted according to the age. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publication 85–23, revised 2011). All protocols were approved by the institutional review board for animal experiments of the Faculty of Veterinary Medicine, Benha University, Egypt with ethical approval number (BUFVTM 09-08-22).

*2.2. Tested substance:*

SB is the sodium salt of butyric acid has general formula Na ($C_3H_7COO$). SB was purchased from Sigma-Aldrich (St. Louis, MO, USA). SB is usually found as white, coated, crystalline solid water-soluble salt. SB have been weighted dose 1g/Kg feed and thoroughly mixed with the basal ration for groups that treated with SB.

*2.3. Materials used for immunological studies:*

Parts from the intestine (ileum and jejunum) were collected and preserved in to10% neutral – buffered formalin. Trizol® reagent from Invitrogen (Carlsbad, CA, USA) used for RNA extraction. SYBR®Green PCR Master Mix (Applied bio systems, USA) for reverse transcription-quantitative polymerase chain reactions (RT-qPCR).7500Real –Time PCR system (Applied Bio systems, USA) for reactions performance eppendorf tubes 1.5 ml capacity. Monochannel micropipettes 20-200µI, 100-1000µI (biohit), sterile filter tips (200µI, 1000µI) capacity. Centrifuge (sigma Sartorius) and Sterile glass beads.

*2.4. Materials used for histopathological studies:*

Buffered formalin solution (10%) for tissue samples fixation, alcohol for sample dehydration and rinsing, Paraffin, Leica rotator microtome (RM2145, Leica Microsystems, Wetzalar, Germany) for sample cutting, Hemotaxylin and eosin stain (H&E) for staining, Image J analysis software (National Institutes of Health, MD, USA) for histophotometric analysis, sterile glass slides and cover slides, and microscope have been used.

*2.5. Experimental design:*

One-hundred-day old broiler chicks were used in this study. The chicks were reared for a total period of 37 days in a standard environmental and nutritional conditions. Chicks were fed on balanced commercial ration that formulated to satisfy adequate requirements of all nutrients according to NRC (1994). Chicks were vaccinated against Gumboro and Newcastle diseases.

Broiler chicks were weighed at the age of one day old and randomly divided into four groups, 25 chicks per each, and subjected to the following different treatments:G1, chicks were fed on basal ration with no additives and without infection; G2, chicks were fed on basal ration and infected with *Eimeria* (1.0 ml solution containing $5.0 \times 10^3$ sporulated *E. maxima* oocysts)on the 22$^{nd}$ day; G3, chicks were fed on basal ration enriched with SB (1gm/kg ration)and infected with *Eimeria* (as G2); G4, chicks were fed on basal ration enriched with SB (as G3).

*2.6. Parasite passage:*

Four weeks old Lohmann Selected Leghorn (LSL) chickens were raised under specific pathogen-free conditions were used for *E. maxima* oocysts propagation as previously described (Long *et al.*, 1976). Standard methods were used to recover sporulated oocysts and to purify sporozoites through nylon wool (Shirley *et al.*, 1995, Pastor-Fernández *et al.*, 2019).

*Oral Infection with E. maxima*

All chickens in infected groups (G3 and G4) were infected by oral gavage at 21 D of age with $5.0 \times 10^3$ oocysts of *E. maxima*, as previously described (Lillehoj *et al.*, 2016, Oh *et al.*, 2018).

*2.7. Sampling:*

Five chickens per group were euthanized on the 5$^{th}$, 10$^{th}$, and 15$^{th}$ days post-infection (viz on the 27$^{th}$, 32$^{nd}$, 37$^{th}$; respectively) tissue samples were collected.

Parts from intestine (jejunum and ileum) were collected, washed with normal saline, and kept in 10% formalin solution for histopathological examination.

*2.8. Immunological assessment:*

*QRT-PCR*

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to analyze the expressions of gens under investigation. Total RNA from the intestine was extracted by using the TRIzol® reagent (Invitrogen, Carlsbad, CAUSA). Then, reverse-transcribing of the extracted RNA was done to prepare cDNA. Table (1) shows the employed primer sequences of the different cytokine genes. RT-qPCR were completed by Power SYBR® Green PCR Master Mix (Applied Bio systems, USA). Reactions were done by the 7500 Real-Time PCR System (Applied Bio systems, USA). Thermal cycles were done at 95°C for 4 minutes, 40 cycles of 10 seconds at 95°C, 30 seconds at 60°C, and 10 seconds at 72°C. Data were given as relative fold changes compared to the control gene expression.

*2.9. Histopathological and Morphometrical assessment of intestinal mucosa:*

About 3.0 cm segment from jejunum was dissected out. The tissues were put in 10% neutral-buffered formalin solution for three days for fixation. then, samples were dehydrated then rinsed several times in absolute alcohol, then submersed in paraffin. Serial 5-µm longitudinal sections were cut on Leica Rotary Microtome (RM 2145, Leica Microsystems, Wetzlar and Germany) and mounted on glass slides. Then, slides stained with hematoxylin and eosin (H&E).

The histomorphometric analysis was done using Image J analysis software (National Institutes of Health, MD, USA), where's the crypt depth measured from the crypt-villus junction to the base of the crypt, villus height measured from the tip of the villus to the villus- crypt junction and villus width measured from the mid of the villus.

*2.10. Statistical analysis:*

Statistical analysis was performed by SPSS (version20.0; SPSS Inc, Chicago, USA). One way ANOVA was conducted on different parameters using Tukey HSDa followed by post hoc. Results were expressed as mean ± SEM. The mean difference is significant at the level $P \leq 0.05$.

Table 1 Primer sequences used for RT-qPCR.

| Gene | Primers (5-3) | Accession No. |
|---|---|---|
| β –actin | F: ACCTGAGCGCAAGTACTCTGTCT<br>R: CATCGTACTCCTGCTTGCTGAT | NM_205518.1 |
| IL 10 | F: ACATCCAACTGCTCAGCTCT<br>R: ATGCTCTGCTGACTGGT | NM_001004414.2 |
| *IFN-γ* | F: GAACTGGACAGAGAGAAATGAGA<br>R: ATGTGTTTGATGTGCGGCTT | NM_205149 |
| *IL-1β* | F: CAGCCTCAGCGAAGAGACCTT<br>R: CACTGTGGTGTGCTCAGAATCC | XM_015297469.1 |

shows the employed primer sequences of the different cytokine genes, R, reverse primer, F, forward primer, β- actin, beta actin, IL-1β, interleukin-1β, IL-10, intrlleukin-10, INF-γ, interferon gamma

## 3. RESULTS

*Effect of SB on intestinal histomorphology:*

*Gross appearance:*

G1 showed intestinal lesion like petechial hemorrhage, mucoid blood-tinged exudates but G3showed milder signs.

*Histopathological examination:*

Jejunum and ileum in G1 showed normal intestinal villi. G2 showed enteritis associated with invasion of the epithelial lining with different stages of *Eimeria* (figure 1). Signs were more severe at 10 days post infection; where, jejunum showed necrotic enteritis with sloughing of the epithelia. While these findings became mild to moderate at 15 days post infection. G3showed catarrhal enteritis associated with cystic crypts with fewer number of *Eimeria* stages within the mucosal lining of the crypts. At 10 days post infection, there was marked decrease the enteritis features with noticeable intestinal epithelial regeneration. While, fifteen days post infection there were marked increase the intestinal epithelia regeneration with mild mononuclear inflammatory cells infiltration within the mucosa (figure 2).

*Morphometrical result of intestinal villi and crypt:*

There was significant decrease ($P \leq 0.05$) in intestinal crypt depth, villi length and width in G2comparedwith G1. There was significant increase in villi length, width, and crypt depth in G3compared to G2. There were increase in intestinal crypt depth and villi length in G4 compared to G1 control group. Table (2) showed the intestinal villi and crypt parameters difference between different groups.

*Role of SB on inflammatory and immune response:*

There was marked up regulation of INF- γ and IL-1β and down regulation of IL-10 in G2, while G3 showed significant decrease the expression of INF-γ and IL-1βand increase in IL-10 gene expression as shown in figure (3, 4, 5).

Table 2 Morphometrical results of intestinal villi parameters of normal and Challenged birds with coccidia (E.maxima).

| Groups | Villi length | Villi width | Crypt depth |
|---|---|---|---|
| 1st euthanasia | | | |
| G1 Control | 865.11±58.10 | 94.01± 10.09 | 185.70± 9.59 |
| G2 Control +ve | 529.72±38.19### | 105.11± 22.11 | 84.80± 9.41### |
| G3 Infected treated | 711.50±70.81*** | 75.10± 6.61*** | 172.90± 14.01*** |
| G4 SB | 1001.90±39.68 | 94.70±14.89 | 215.01± 8.11 |
| 2nd Euthanasia | | | |
| G1 | 1003.90± 82.81 | 81.40± 7.26 | 204.41± 19.50 |
| G2 | 613.10± 70.47### | 96.01± 21.16### | 90.80± 20.56### |
| G3 | 961.90± 34.37*** | 86.70± 24.62*** | 208.80± 9.10*** |
| G4 | 1240.90± 50.36 | 84.21± 9.39 | 263.70± 20.92 |
| 3rd euthanasia | | | |
| G1 | 1144.01± 42.98 | 77.40± 11.74 | 220.70± 17.55 |
| G2 | 664.20± 39.17### | 105.51± 12.88### | 168.10± 16.10### |
| G3 | 1160.70± 66.82*** | 104.40± 5.56*** | 229.61± 18.43*** |
| G4 | 1372.51± 62.61 | 79.32± 8.75 | 329.00± 27.25 |

Data are expressed as mean±SED, # indicates significance in comparison with control group and * indicates significance in comparison with control positive group ($P \leq 0.05$).

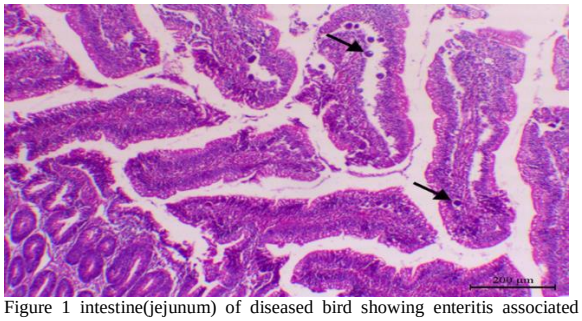

Figure 1 intestine(jejunum) of diseased bird showing enteritis associated with different stages of *Eimeria*(arrows)within the intestinal mucosa, H&E stain.

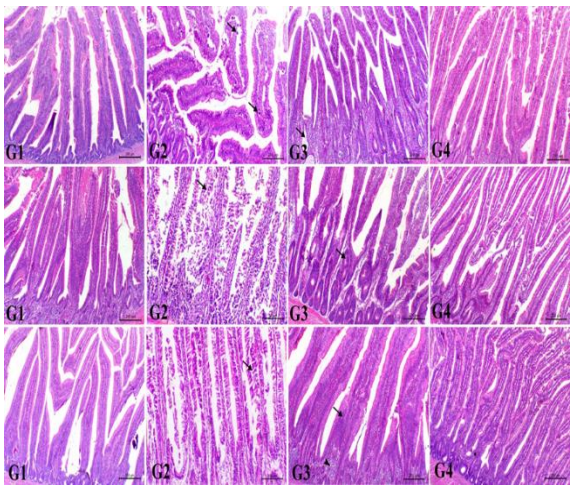


Figure 2 Histopathological changes (jejunum) of different treated groupsat five, ten and fifteen days post infection with *E.maxima*.; G1 con, G2 infected, G3 infected treated and G4 treated. G 1 showing normal intestinal villi, G2 showing catarrhal and necrotic enteritis associated with parasitic stages (arrows), G3 showing decrease the intestinal inflammation and G4 showing marked elevation in the intestinal villi length. H&E stain, bar= 200 µm.

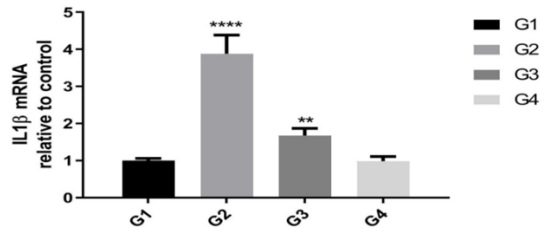


Figure 3 over expression of IL-1β in G2 whileG3 moderate expression.

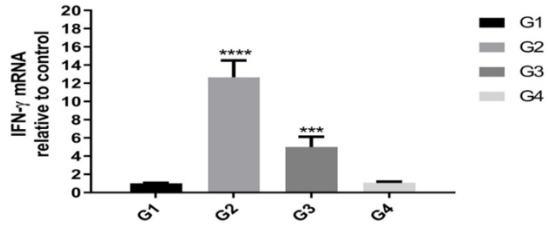


Figure 4 over expression of INF-γ in G2 while G3 moderate expression.

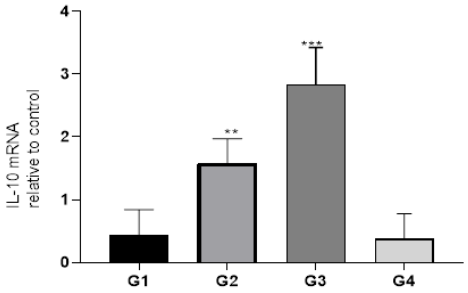


Figure 5 over expression of IL-10 in G3 while G2 moderate expression.

## 4. DISCUSSION

More studies were examined role of SB as alternative to antibiotic that can be used as feed additive and study the effect of SB toward pathogenic bacteria in poultry such as *clostridium perfringens* and *salmonella typhimurium* (NamKung et al. 2011). BA has antibacterial effect and protective effect since it elevates intestinal cell integrity and increase intestinal cell resistance to immunological stressors prevention of *salmonella enteritidis* and *E. maxima* infection and can protect intestinal cell from necrotic enteritis (Ali et al. 2014; Liu et al. 2019). In the present study *E. maxima* have been used for inducing infection in broiler since *E. maxima* works mainly on small intestine Jejunum and ileum and complete life cycle in the intestinal mucosa inducing inflammation, gut leakiness, and histological changes. In the current study there were mild signs of *E. maxima* that have been indicated the role of SB as antimicrobial. Guilloteau et al. (2010) found that SB provides intestinal epithelial cells with energy that increase proliferation differentiation of the epithelial cells. Butyrate seems to stimulate development of intestinal epithelium since it stimulates duodenal mucosa growth (Hu and Guo 2007). Elnser et al. (2019) found that there was better growth of quail intestinal villi ingroup feed on SB compared to control negative group and he explained that were due to the role of butyrate in stimulating intestinal blood flow and increase peptides secretion that enhance enterocytes proliferation, increase villi length, and repair damaged mucosa. These findings may interpret the increase in villus length in the present study as there were increase in intestinal crypt depth and villi length between control and treated groups.

SB modifies the immune and inflammatory response.SB has anti-oxidative, anti-inflammatory, antimicrobial and immunomodulatory function (Zhang et al. 2015). In the present study there were down regulation of pro inflammatory gene expression INF-γ and IL-1βand up-regulation of IL-10 agree with Andoh et al. 2010 found that SB has immunomodulatory properties by inhibition of nuclear factor kappa B there by inhibit chemokine gene expression and pro inflammatory response.

Butyrate stimulates the intestinal mucosa growth (Hu and Guo 2007; Zhang et al.,2011) this explain the intestinal epithelium development in present study as showed in table (2) there were increase in crypt depth and villi length agree with Lesson et al. (2005). Butyrate stimulates the intestinal epithelial cells proliferation and differentiation and maintains the villus structure since it acts as a source of energy for enterocyte (Kinoshita et al. 2002).

## 5. CONCLUSION

The current study demonstrated that sodium butyrate has role in attenuating intestinal inflammation in broiler infected with *E. maxima* indicated by moderate signs of coccidiosis (intestinal hemorrhage, intestinal lesion and histological scores in the jejunum and ileum) and modifying inflammatory cytokines genes expression.SB improves the intestinal mucosa and increases villus length and crypt depth and thus improve gut health. SB seems to be optimized for intestinal immune response in broiler at higher dose 1gm/kg feed.

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