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Research Article

Effect of Na-butyrate Supplementation on Electromicroscopy, Virulence Gene Expression Analysis and Gut Integrity of Experimentally Induced *Salmonella enteritidis* in Broiler Chickens

[1]M.H.H. Awaad, [1]Wafaa A. Abd El-Ghany, [3]S.A. Nasef, [4]Medhat S. El-Halawany, [2]Faten F. Mohamed and [3]Ahmed F. Gaber

[1]Departement of Poultry Diseases, Faculty of Veterinary Medicine, Cairo University, Egypt
[2]Departement of Pathology, Faculty of Veterinary Medicine, Cairo University, Egypt
[3]Animal Health Research Institute, Dokki, Cairo, Egypt
[4]Departement of Zoology, Faculty of Science, Cairo University, Egypt

Abstract

Objectives: The efficacy of microencapsulated Na-butyrate (NaB) supplementation for reducing *Salmonella enteritidis* (*S. enteritidis*) infection was investigated. **Methodology:** One day old broiler chicks (n = 200) were allotted into 4 equal groups. At 2nd day of age, birds of groups 3 and 4 were challenged by crop gavage with 5×10^8 CFU mL^{-1} of *S. enteritidis*. Groups 2 and 3 were supplemented with NaB diet while 1 and 4 were fed on a plain ration without treatment. Scanning Electron Microscopy (SEM) of re-isolated *S. enteritidis* of challenged NaB treated broilers revealed broken cells with morphological changes in comparison with untreated challenged group, however, Transmission Electron Microscopy (TEM) showed several lipids like bodies that barely detected in untreated group. **Results:** Results from Polymerase Chain Reaction (PCR) analyses revealed that *S. enteritidis* strain from untreated challenged control harbored virulence genes *inv*A, *stn* and *pef*A with incidence rate of 100%. While, NaB supplementation resulted in detection of 100% of *inv*A gene and only 66.6% of *pef*A and *stn* genes. Gut integrity of NaB supplemented groups showed significant ($p \leq 0.05$) increase in villi height and villus height: crypt depth ratio and had positive effect in maintenance of healthy intestinal epithelial cells. **Conclusion:** NaB supplement is effective in reducing *S. enteritidis* in chickens.

Key words: *Salmonella*, butyrate, chickens, PCR, electron microscopy

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Corresponding Author: Wafaa A. Abd El-Ghany, Department of Poultry Diseases, Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The importance of gastrointestinal tract health in broiler chickens has been increasingly recognized due to its contribution to their overall health and performance[1]. *Salmonellae* infections can cause significant growth depression or even mortality in young birds. Salmonellosis is a zoonotic bacterial disease of significant importance[2]. *Salmonella enteritidis* (*S. enteritidis*) is one of the main *Salmonella* serovars involved in human food poisoning outbreaks and sometimes deaths[3]. Organic acids are among the alternative antibiotic growth promoters that have already being used in practice for decades to stimulate growth performance in poultry[4-6]. Amongst these organic acids, Short Chain Fatty Acids (SCFA) are considered as potential alternatives[7]. Butyric acid is one of these SCFA which has higher bactericidal activity when the acid is undissociated[8,9]. Accordingly, it can be used for the treatment of several intestinal bacterial infections like salmonellosis[10].

The present trial was conducted to elucidate the effects of usage of sodium butyrate (NaB) encapsulated in palm fat on intestinal enumeration, electomicroscopic morphology, virulence genes and intestinal histomorphometry of experimentally induced *S. enteritidis* infection in broilers.

MATERIALS AND METHODS

Na-butyrate (NaB): That product of Na-butyrate encapsulated in palm fat(Admix®30) (produced by NUTRI-AD International, Belgium) was used in this trial in the following dietary levels in the test group; starter diet 1 kg t^{-1}, grower diet 0.5 kg t^{-1} and finisher diet 0.25 kg t^{-1}.

Experimental birds: One day-old cobb broiler chickens (n = 205) were used in this study. Chick box paper liners as well as organs of 5 sacrificed chicks were cultured to screen for presence of *Salmonellae*. The remaining birds were allotted into 4 equal groups (1-4). Each group divided into 5 replicates consisting of 10 birds each. The chickens were vaccinated against different diseases according to the vaccination programs usually adopted in Egyptian chicken broiler farms. The commercial diets used were formulated to meet the nutrient requirements of the broiler chicks during starter, grower and finisher periods according to the National Research Council[11]. Feed and water were given *ad libitum*.

Experimental design: The experiment was carried out according to the National Regulations on Animal Welfare and approved by Institutional Animal Ethical Committee, Faculty of Veterinary Medicine, Cairo University. Chickens of groups 2 and 3 were supplemented with NaB diet, while groups 1 and 4 fed on a plain ration without treatment. At 2nd day of age, birds of groups 3 and 4 individually infected by crop gavage with 5×10^8 CFU mL^{-1} of *S. enteritidis*[12]. The challenge inoculate checked for purity and confirmed by PCR. Chickens of group 1 were kept without infection or NaB supplementation as blank controls. At 7th and 19th day of age, 5 birds/group were sacrificed and subjected to bacteriological examination for *S. enteritidis* re-isolation from caecum and liver. At the end of the experiment (35 days), 5 caecal contents from each group were examined for *S. enteritidis* enumeration (colonization)[13].

Electron microscopy examination: At 17th day of age, caecal specimens of 3 birds of groups 3 and 4 were similarly subjected to *S. enteritidis* re-isolation and the isolated strains were subjected to electron microscopy examination for detection of any morphological alteration. In SEM, *S. enteritidis* randomly selected colonies (isolated from group 3 and 4) cultured on agar medium were first excised and trimmed, then fixed by 2.5% glutaraldehyde. The samples were dehydrated by serial dilution of ethanol and then dried using Audissamdri-815 CO_2 critical point drier (Tousimis Inc., Rockville, MD, USA). The samples were coated by gold sputter coater (SPI-module) and examined using JOEL-JSM 5500 (JEOL Ltd., Tokyo, Japan) scanning electron microscope was adopted at 20 kV at the Regional Center of Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. The TEM was performed using standard procedures. Briefly, grown *S. enteritidis* cells (isolated from 3 and 4) at mid-log phase were harvested by centrifugation (4000×g for 10 min) and fixed in 2.5% glutaraldehyde in PBS (pH 7.4). This was followed by fixation in 1% osmium tetroxide and then dehydrated by serial dehydration in ascending ethanol rinses. Thin sections of epoxy resin-embedded specimens were imaged on a JEM-1400 (JEOL Ltd., Tokyo, Japan) operated at accelerating voltage of 80 kV at the electron microscopy unit, Cairo University Research Park (CURP), Giza, Egypt.

Detection of *S. enteritidis* virulence gene expression using conventional PCR: The DNA of the isolated bacterial strains was extracted and amplified for virulence genes (*inv*A, *pef*A and *stn*). *Salmonella enteritidis* DNA was extracted using commercially available kit, QIAamp DNA Mini Kit, (Cat. # 51304) according to the manufacturer's instructions. Virulence genes were amplified using their specific primers[14,15]. The amplification products were photographed by a gel documentation system and the data was analyzed through computer software.

Gut integrity assay (histomorphometric assay): At 35 days of age, 3 birds from groups 1-4 were sacrificed and small intestine were collected and immediately immersed in 10% buffered formalin. After fixation, 1 cm-thick samples were taken from the jejunum and ileum. Routine histological laboratory methods including dehydration, clearing and paraffin embedding were used and paraffin blocks were made. The slides stained by haematoxylin and eosin[16]. Histological indices were measured using digital photography and light microscopy. The villus height was measured from the apical to the basal region, which corresponds to the superior portion of the crypts. Crypts were measured from the basis until the region of transition between the crypt and the villus.

Statistical analysis: One-way analysis of variance adopted using SAS software general liner models procedure[17]. The main factor was NaB supplementation as a mean effect. Mean values assessed for significance using Duncan's multiple range tests[18].

RESULTS AND DISCUSSION

In the current study, re-isolation of *S. enteritidis* from liver and caecum of experimentally infected chickens at day 19th of age revealed 0% in NaB supplemented group as compared with 80% in infected untreated broilers. Reduction in *S. enteritidis* enumeration in the caecum of NaB supplemented group at 35th day of age (a mean of 10^1 and 1.1×10^4 CFU g^{-1} in NaB supplemented and positive control groups, respectively) was important for the microbiological safety of poultry products, as this site and cloaca represent 2 common locations in the birds where the bacteria are present in high numbers[19]. This might be due to the continuous slow release of the acidifier[20]. These results are in agreement with the findings of Cox *et al.*[21], who showed that butyric acid in particular was effective in reducing *Salmonella* colonization of the intestine and Van Immerseel *et al.*[7,10], who found that coated butyric acid was superior to uncoated butyric acid in reducing *Salmonella* colonization of the caeca and internal organs of chickens shortly after infection with *S. enteritidis*. Moreover, Zou *et al.*[22] reported that the populations of *Salmonellae*, *Escherichia coli* and *Clostridium perfringens* in the caecum were decreased by supplementation of NaB.

Either scanning or transmission electron microscopy (SEM) or (TEM) examination of *S. enteritidis* strains re-isolated from the caeca of broiler chickens (with or without NaB supplementation) revealed some changes, according to the conditions of examination. Figure 1 indicates that SEM of samples isolated from challenged NAB treated birds revealed some degraded and broken cells and the bacterial population looked aged for some extent. While the bacterial cells looked healthy in control group. The TEM of samples isolated from treated group showed transparent lipids like bodies, while normal features were observed in the untreated group (Fig. 2). Further investigations are necessary to understand the association between NaB treatment and detected changes in bacterial cells.

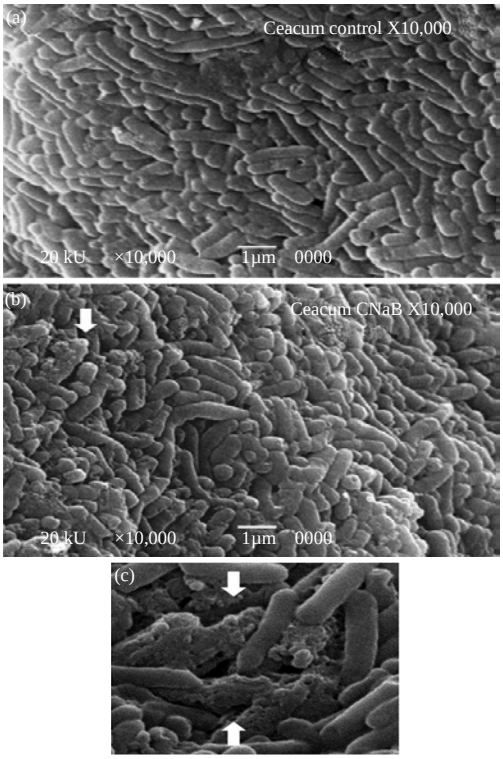

Fig. 1(a-c): Scanning Electron Microscopy (SEM) morphological features of examined *Salmonella enteritidis* isolated from the caecum of chicken broilers after 17 days of infection, (a) SEM of *Salmonella enteritidis* colonies re-isolated from the caecum of experimentally infected broilers without NaB supplementation (positive control group) and (b and c) SEM of *Salmonella enteritidis* colonies re-isolated from the caecum of experimentally infected broilers supplemented with NaB, notice the white arrows showing remnants of broken cells

The pathogenesis of salmonellosis depends upon a large number of factors controlled by an array of genes those synergies into the actual virulence of *Salmonella*[15]. Nucleic acid based diagnostic techniques are being employed for the

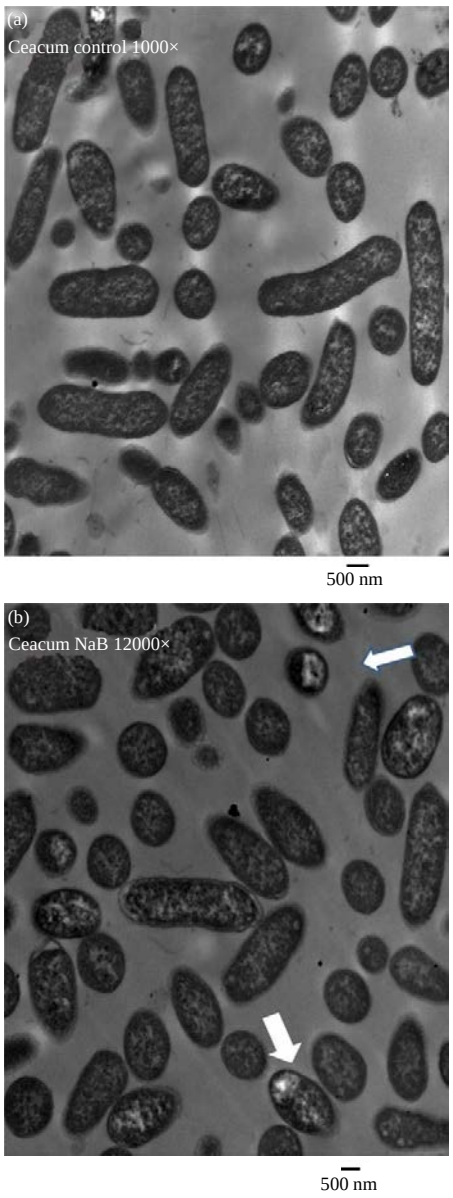

Fig. 2(a-b): Transmission Electron Microscopy (TEM) morphological features of examined *Salmonella enteritidis* isolated from the caecum of chicken broilers after 17 days of infection, (a) TEM of thin sections of *Salmonella enteritidis* colonies re-isolated from the caecum of experimentally infected broilers without NaB supplementation (positive control group) and (b) TEM of *Salmonella enteritidis* colonies re-isolated from the caecum of experimentally infected broilers supplemented with NaB, notice the transparent bodies are evident (white arrows). Scale bar = 500 nm

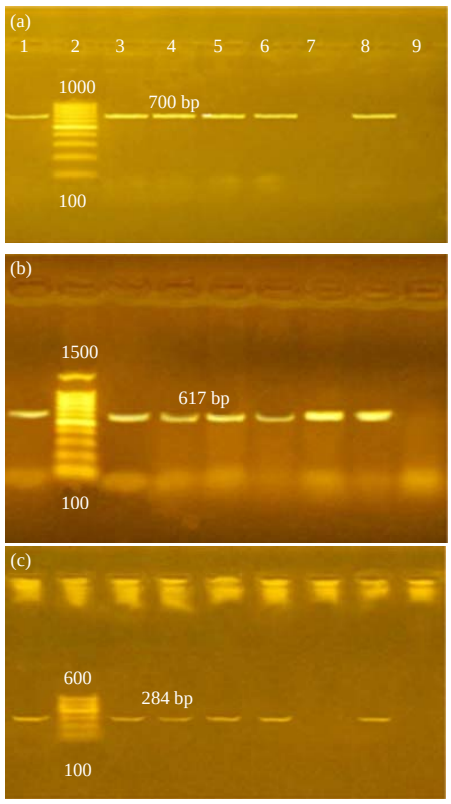

Fig. 3(a-c): PCR results, (a) *pef*A gene: Positive amplification of 700 bp fragment was detected in three samples of group 4 (positive control) and two samples of groups 3 (NaB supplemented), (b) *stn* gene. Positive amplification of 617 bp fragment was detected in three samples of both groups 3 (NaB treated) and 4 (positive control) and (c) *invA* gene: Positive amplification of 284 bp fragment was detected in two samples of group 3 (NaB treated) and three samples of group 4 (positive control). Lane 1: Positive control (*Salmonella eteritidis* primary inoculum). Lane 2: Ladder. Lanes 3, 4, 5: Group 4 (Positive control *Salmonella enteritidis* reisolated from infected chickens). Lanes: 6,7, 8: Group 3 (*Salmonella enteritidis* reisolated from infected chickens treated with NaB). Lane 9: Negative control (*E. coli*)

detection of various gene-encoded virulence factors[23,24]. Accordingly, *in vivo* assay using conventional PCR was conducted to express these genes of *S. enteritidis* strain after its re-isolation from livers of experimentally infected birds at 7th day post infection from treated or untreated groups with NaB. Three virulence genes were assayed including, *Salmonella* invasion (*inv*A) *Salmonella* enterotoxin (*stn*) and plasmid encoded fimbrial (*pef*A) genes. In Fig. 3, *S. enteritidis* strains that re-isolated from untreated group (group 4) detected all these genes with an incidence of 100%. While modulation and deletion of *pef*A or *stn* genes were recorded in NaB treated strains (group 3) with an incidence of 33.33%.

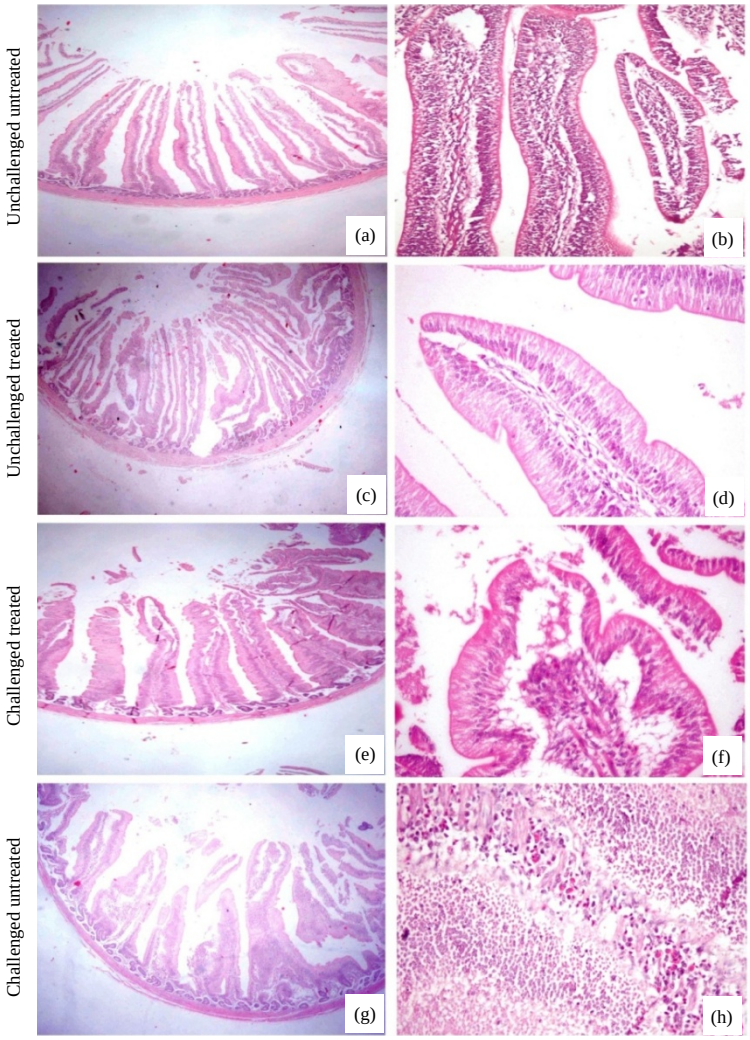

Fig. 4(a-h): Small intestine of broiler chickens (a, c, e, g) Demonstrating the histomorphology of intestine in unchallenged untreated group, unchallenged NaB treated group, *Salmonella enteritidis*, challenged NaB treated group and *Salmonella enteritidis* challenged untreated group (X40), (b) Unchallenged untreated group showing normal histological structure on intestinal villi (X200) and (d) Unchallenged NaB treated group showing intact normal histological structure of enterocytes and lamina propria (X400), (f) *Salmonella enteritidis* challenged NaB treated group showing intact enterocytes with increased number of goblet cells and folding of intestinal surface with mild inflammatory reaction involving the lamina propria (X400), (h) *Salmonella enteritidis* challenged untreated group showing severe necrosis of enterocytes with massive heterophiles infiltration in lamina propria (X400)

Durant *et al.*[25] reported that SCFA modulate the expression of the *hil*A and *inv*F genes of *S. typhimurium*. Boyen *et al.*[26] found that some frequently used SCFA and medium-chain fatty acids were able to alter virulence gene expression and decrease *S. typhimurium* colonization and shedding in pigs using well established and controlled *in vitro* and *in vivo* assays. Modulation of these virulence genes in the present work might explain the antibacterial effect of NaB, a conclusion that could be partially confirmed by reports of Baloda *et al.*[27] and Chopra *et al.*[28] who mentioned that *Salmonella* induced diarrhoea was a complex phenomenon involving several pathogenic mechanisms including production of enterotoxin which was mediated by *stn* gene. Thorns *et al.*[29] have shown that *pef* genes product play an important role in bacterial adhesion to the epithelial cells. Also, Csiko *et al.*[30] found that butyrate, a commonly applied feed additive in poultry nutrition, could modify the expression of certain genes, including those encoding cytochrome P450 (CYP) enzymes.

Results of histopathological examination are seen in Table 1 and Fig. 4. *Salmonella enteritidis* challenged untreated group showed severe necrosis of enterocytes with

Table 1: Histomorphometric analysis of jejunal intestinal segments in different treated chicken groups

Group No.	NaB treatment	*Salmonella enteritidis* infection	Villi height (μm)	Crypt depth (μm)	Villus height: crypt depth ratio
1 Blank control	-	-	644.36±13.0^{c}	69.95±1.88^{ab}	9.18±0.28^{bc}
2 Negative control	+	-	783.99±18.5^{d}	80.83±2.43^{b}	9.95±0.35^{c}
3 NaB	+	+	585.29±12.3^{b}	62.91±3.33^{a}	9.35±0.43^{bc}
4 Positive control	-	+	517.50±20.7^{a}	102.38±7.53^{c}	5.72±0.63^{a}

NaB: Sodium butyrate, Different superscripts are significantly different ($p<0.05$) and results are reported as Mean±SEM

massive heterophiles infiltration in lamina propria. On the other hand, challenged NaB treated group showed intact enterocytes with increased number of goblet cells besides folding of intestinal surface with mild inflammatory reaction involving the lamina propria. Brouns *et al*.[31] and Pryde *et al*.[32] mentioned that butyrate appeared to play a role in the development of the intestinal epithelial cells. Jiang *et al*.[33] investigated the effects of micro-encapsulated sodium butyrate on oxidative stress and apoptosis induced by dietary corticosterone in the intestinal mucosa of broiler chickens. The protective effect of NaB supplementation in birds challenged with *S. enteritidis* was reflected on intestinal histomorphometric parameters as the presence of microbial load like *S. enteritidis* caused significant reduction in villous height, villous height: crypt depth ratio and significant ($p \leq 0.05$) increase in crypt depth that is not related to increase villous height as shown but related to increased cell turn over and sloughing induced by *Salmonella*. Accordingly, NaB has a protective effect against fast tissue turn over induced by *S. enteritidis*, relatively and partially increased villous height and villous height: crypt depth ratio as compared with NaB unchallenged group (group 2) that achieving significant ($p \leq 0.05$) increase in intestinal parameters and the final results reflected on villous height: crypt depth ratio to be similar to uninfected untreated group (normal histological structure of intestinal villi) (group 1). These results indicated that NaB supplementation achieved a beneficial effect on intestinal histomorphomatric parameters by increasing villous height and villous height: crypt depth ratio, which in turn positively might reflect on nutrient digestion and absorption and finally the body weight. Also, in group 2, NaB supplementation increased crypt depth that was beneficial whereas crypt depth is considered as a progenitor cells for villous epithelium and this may be assumed to be related to the demand for increase of the villous height and hence total increase in gut surface area. Mallo *et al*.[34] concluded that inclusion of butyrate in the diet improves the digestibility of energy and protein by increasing intestinal absorption surface. Chamba *et al*.[35] mentioned that jejunal villi of birds fed sodium butyrate and colistin at 42 days were higher than those in birds fed the control diet.

CONCLUSION

In conclusion, the used microencapsulated NaB in the present investigation had a bacteriocidal action against *S. eneteritidis* as it reduced its enumeration in cecum and liver and altered its morphology as well as modulated some of its virulence genes. It also played a positive role in the development of intestinal epithelial cells.

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