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Effects of dietary sodium butyrate supplementation on the intestinal morphological structure, absorptive function and gut flora in chickens☆

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Abstract

A study was carried out to investigate the effects of dietary sodium butyrate (SB) on the intestine and growth performance in chickens. Three hundred and thirty six day-old AA broiler chicks were allocated at random into four groups with six replicates each. Four groups were fed with basal diet (control) or diets supplemented with SB at the level of 500, 1000, 2000 mg/kg. Body weight gain increased linearly during the period from 0 to 21 days as the dietary supplementation of SB increased ($P<0.05$). Dietary supplementation of SB influenced feed conversion ratio (FCR) in a positive quadratic fashion during the period from 0 to 42 days ($P<0.05$). Dietary SB did not influence the absorptive function of jejunum ($P>0.05$). The concentrations of DNA, RNA, and protein in duodenal mucosa showed negative quadratic responses to the increase of dietary SB supplementation level ($P<0.05$). The ratio of villus height to crypt depth increased linearly with the increase of dietary SB supplementation ($P<0.01$). The *Lactobacillus* count decreased linearly with the increase of dietary SB supplementation ($P<0.01$).

Keywords: Butyrate; Chicken; Intestinal morphological structure; Absorptive function; Digestive microflora

Abbreviations: SB, sodium butyrate; SCFAs, short-chain fatty acids; FCR, feed conversion ratio

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1. Introduction

Short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate are produced in the cecum and colon of animals via the fermentation of carbohydrates such as dietary fiber and unabsorbed starch. Numerous studies have been on effects of butyrate on colon through total parenteral nutrition, intestine perfusion and bacterial fermentation. SB can induce absorption of water and sodium and proliferation of intestinal cells (Kripke et al., 1989; Friedel and Levine, 1992), be used as energy resources (Jozefiak et al., 2004), and stimulate intestinal blood flow and the synthesis of gastrointestinal hormones (Mortensen et al., 1990; Mineo et al., 1994).

Microencapsulated butyrate can influence the hind gastrointestinal tract (Van Immerseel et al., 2004). Non-protected SCFAs can have effect on the upper part of the digestive tract, but not directly further down (Bolton and Dewar, 1965; Hume et al., 1993; Thompson and Hinton, 1997). Although studies have been published on the effect of various organic acids on performance in poultry, very little is known on intestinal effects. The current experiment was designed to study whether SB supplementation in the diet influences morphological structure, absorptive function and gut flora of the intestine of broiler chickens.

2. Materials and methods

2.1. Experimental procedures

Three hundred and thirty six day-old healthy AA broiler chicks were randomly allocated into 4 groups with 6 replicates of 14 birds. The birds were reared in cages. The house was controlled at constant temperature and maintained on a 24 h, constant-light regime. The birds were allowed *ad libitum* access to the food and water, and were fed with one of four diets, basal diets (control) or diets supplemented with SB at level of 500, 1000, 2000 mg/kg for 6 weeks. The composition of the basal diet is shown in Table 1.

Chickens were weighed on day 21 and day 42 and feed consumption in each cage was recorded at the same time. Feed conversion ratio (FCR) (g feed/g gain) was calculated during each period. Daily mortalities were recorded.

On the day 21, one chicken was slaughtered from each replicate. After exsanguination from the jugular vein, the small intestine was removed. Contents of the jejunum (from the end of the duodenum to Meckel's diverticulum) were used to examine SCFAs levels and microbial population. The 2 cm jejunum from Treitz ligament was fixed with buffered formalin for morphological examination. A 10 cm central duodenum segment and 10 cm central jejunum segment were used for the measurement of the concentration of DNA, RNA, and protein in mucosa. The fore 10 cm jejunum was used for absorption assay.

2.2. Microbiological examination

All samples were diluted 1:10 with normal saline solution and their weight was recorded. Further serial dilutions (10^{-2} to 10^{-5}) were made in normal saline and small volume samples (100 μl) were plated on agar plates. *E. coli* was quantified on the eosin-methylene

Table 1
Composition of basal diet (g/kg)

Ingredients	0–3 weeks	4–6 weeks
Maize	603.0	661.7
Soybean	332	280
CaHPO4	18	13
Limestone	13	14
NaCl	3.5	3.5
Soybean oil	25	23
Lysine HCl	0.3	0.5
Methionine	2	1.1
Vitamin premix[a]	0.2	0.2
Mineral premix[b]	2	2
Choline chloride	1	1
Nutrient level		
Metabolizable energy (MJ/kg)[c]	12.32	12.53
Crude protein (g/kg)	210	190
Lysine (g/kg)	11	10
Methionine(g/kg)	4.9	3.8
Calcium (g/kg)	10	9
Available phosphorus (g/kg)	4.5	3.6

[a] Vitamin premix provided 1 kg of diet with: vitamin A, 10,800 IU; vitamin D_3, 2160 IU; vitamin E, 15 IU; vitamin K_3, 1.0 mg; vitamin B_1, 4 mg; riboflavin, 5 mg; pantothenic acid 10 mg; niacin, 25 mg; vitamin B_6, 8 mg; folic acid, 0.4 mg; vitamin B_{12}, 0.08 mg; biotin, 0.15 mg.
[b] Mineral premix provided 1 kg of diet with: I, 0.35 mg; Se, 0.15 mg; Zn, 40 mg; Cu, 8 mg; Fe, 80 mg; Mn, 100 mg.
[c] Metabolizable energy was obtained by calculation.

blue agar (components: peptone, 10 g; sodium eosin, 0.4 g; beef extract power, 5 g; agar, 14.5 g; NaCl, 5 g; lactose, 10 g; methylene blue, 0.065 g; distilled water, 1000 ml) medium, and *Lactobacillus* was counted on the lactic acid agar (components: tomato juice, 50 ml; yeast extract power, 5 g; beef extract, 10 g; lactose, 20 g; glucose, 2 g; K_2HPO_4, 2 g; Tween-80, 1 g; agar, 15 g; distilled water, 950 ml) medium. The results were expressed as $\log_{10}$ cfu/g.

2.3. Analysis of the SCFAs concentrations

The concentration of SCFAs (acetic acid, propionic acid and *n*-butyric acid) was measured using gas chromatography (Zijlstra et al., 1977). 200 mg of chyme were suspended in sterile distilled water (1.6 ml) and metaphosphoric acid (0.2 ml) was added. 2-Mercapto isobutyric acid (0.2 ml) was added as an internal standard. The sample was mixed for 45 min with an orbital shaker and centrifuged for 15 min at 10,000 × *g* at room temperature. One microliter of the extract were injected onto the column of a Hewlett Packard 19091N-213 series chromatograph with a flame ionization detector, a cross-linked phenyl methyl silicone capillary column (30 m × 0.32 mm × 0.5 μm). The carrier gas was helium at 20 ml/min, the air flow was 450 ml/min and the hydrogen flow was 40 ml/min, the injector was heated to 200 °C and the detector to 250 °C.

2.4. Determination of the protein, DNA and RNA concentrations

A 0.5 g mucosa was disrupted in 4 ml distilled water by a microhomogenizer. The prepared homogenate was centrifuged 20 min at 4000 r/min. The supernatant was used for the determination of protein concentration.

Mucosa were disrupted in five volumes of ice-cold 0.1 mol/l NaCl–0.05 mol/l sodium citrate buffer (pH 7.0) using a microhomogenizer, then the mixture was centrifuged 10 min at 4000 r/min. The precipitate was dissolved in five volumes of 0.15 mol/l NaCl–0.1 mol/l Na_2EDTA. 1/4 volume of 5%SDS and NaCl were added into the mixture until the concentration of NaCl was 1.5 mol/l. The mixture was shaken 60 min, then the same volume of chloroform and 3-methyl-1-butanol (24:1 v/v) was added. After shaking 20 min, the mixture was centrifuged 20 min and the water phase was put into beaker. Two volumes of 95% ethanol were added into the beaker. The DNA was wrapped around the glass stick, washed by ethanol (95%) and ether, and dissolved into 0.01 mol/l NaOH solutions for the determination of DNA concentration.

Five milliliters of acetate-acetic acid buffer (0.05 mol/l, pH 5.0) and 1.5 ml SDS (250 g/l) were added in 0.5 g mucosa, and then the mixture was disrupted by a microhomogenizer. The same volume of 80% phenol was added in the prepared homogenate. The mixture was disrupted again by a microhomogenizer and shaken 7 min at 65 °C. Then the mixture was centrifuged 10 min at 3000 r/min. 1/10 volume of 2 mol/l sodium acetate and 2.5 volumes of ice-cold 95% ethanol were added in the supernatant and vortexed 30 s. The mixture was centrifuged 10 min. The precipitate was dissolved in 4 ml distilled water for the determination of RNA concentration.

The protein, DNA, and RNA concentrations were measured by the method of Lowry et al. (1951), the ethidium bromide method (Anderson and Skagen, 1977), the orcinol method (Lin, 1969), respectively.

2.5. Examination of intestinal morphological structure

Samples were fixed in 10% formalin. After fixation, the samples were dehydrated in increasing concentrations of alcohol and embedded in paraffin. Jejunum was sectioned longitudinally in 5 μm thickness. The villus height and crypt depth were determined in longitudinal sections stained with hematoxylin-Van Giesson, using inverted microscope. The measurements were made on six villi and six crypts for each segment.

2.6. Assay of absorptive function

Absorptive function was determined in an *in vitro* system. Preparation was everted sac of chicken jejunum (Wilson and Wiseman, 1954). The sacs containing 2 ml bicarbonate saline with 0.1% glucose were suspended in 50-ml centrifugal tube containing 5 ml bicarbonate saline with 0.1% glucose and 5 ml 10 mmol/l D-xylose solution. The centrifugal tube was fixed in the constant-temperature trough at 37 °C and was gassed with 5% CO_2 and 95% O_2. The sacs were cultured for 1 h, and the centrifugal tube was shaken in every 10 min. The fluids in the centrifugal tube were collected and defined as fluids at the side of mucosa. The concentration of xylose was determined by colorimetry (Eberts, 1979).

2.7. Statistical analysis

The data were analyzed by the general linear models procedure of SAS for analysis of variance. The nature of response to increasing concentration of dietary SB was determined by polynomial contrasts. The model included linear and quadratic contrasts for effects of supplemental SB. When significant differences among diets were found, the significance between the groups of dietary SB supplementation and control was identified by LSD test. A probability of $P<0.05$ was considered for statements of significance.

3. Results

3.1. Growth performance

Body weight gain increased linearly as the dietary supplementation of SB increased during the period from 0 to 21 days (Table 2). SB added to the diet of broilers at the level of 500 or 2000 mg/kg significantly enhanced body weight gain during the period from 0 to 21 days compared to the control ($P<0.05$). SB supplementation had no effects on body weight gain, feed intake and mortality during the period from 0 to 42 days ($P<0.05$). Dietary supplementation of SB influenced feed conversion ratio (FCR) in a positive quadratic fashion during the period from 0 to 42 days ($P<0.05$). FCR was significantly increased with SB at the level of 2000 mg/kg compared to the control ($P<0.05$).

3.2. The concentrations of SCFAs and bacterial count in jejunum contents

Dietary SB did not significantly influence the concentration of SCFAs in jejunal chyme (Table 3). SB supplementation did not significantly influence the number of *E. coli* ($P>0.05$). The number of *Lactobacillus* decreased linearly as dietary SB increased ($P<0.01$), and the number of *Lactobacillus* decreased significantly by SB supplementation at the level of 2000 mg/kg compared to control ($P<0.05$).

Table 2
Effect of dietary sodium butyrate on feed intake, body weight gain, and feed conversion in broilers

	The level of sodium butyrate (mg/kg)				S.E.M.	Linear	Quadratic
	0	500	1000	2000			
0–21 days							
Body weight gain (g)	551	577*	566	577*	4.7	<0.05	NS
Feed intake (g)	849	870	854	883	8.0	NS	NS
FCR	1.54	1.51	1.51	1.53	0.012	NS	NS
Mortality (%)	1.19	2.38	4.76	1.19	0.928	NS	NS
0–42 days							
Body weight gain (kg)	1.65	1.70	1.60	1.60	0.018	NS	NS
Feed intake (kg)	3.24	3.28	3.19	3.26	0.025	NS	NS
FCR	1.96	1.93	2.00	2.05*	0.016	NS	<0.05
Mortality (%)	1.19	3.57	4.76	2.38	0.953	NS	NS

* Means were significantly different compared to the control ($P<0.05$).

Table 3
Effect of dietary sodium butyrate on SCFAs concentration and bacterial count in jejunal chyme

	The level of sodium butyrate (mg/kg)				S.E.M.	Linear	Quadratic
	0	500	1000	2000			
SCFAs concentration (μg/g)							
Acetic acid	198	254	237	205	14.0	NS	NS
Propionic acid	39	37	31	26	2.7	NS	NS
Butyric acid	30	41	39	22	3.7	NS	NS
Bacterial count, $\log_{10}$ cfu/g							
E. coli	5.4	5.8	6.2	5.0	0.32	NS	NS
Lactobacillus	6.7	6.4	6.2	5.4*	0.18	P<0.01	NS

* Means were significantly different compared to the control (P<0.05).

3.3. Xylose absorption by the small intestine and jejunal microscopic morphological structure

Dietary SB supplementation did not significantly influence the concentration of xylose on the side of mucosa (P>0.05) (Table 4). That was to say, dietary SB did not influence the absorption function of jejunum. Dietary SB did not significantly influence the villus height and crypt depth of jejunum (P>0.05). The ratio of villus height to crypt depth increased linearly as dietary SB increased (P<0.01). Compared to the control, dietary SB at the level of 2000 mg/kg significantly enhanced the ratio of villus height to crypt depth (P<0.05).

3.4. DNA, RNA, and protein concentrations in intestinal mucosa

The concentrations of DNA, RNA, and protein in duodenal mucosa showed negative quadratic responses to the increase of dietary SB supplementation level (P<0.05) (Table 5). In contrast to the control, dietary SB supplementation at the level of 500 mg/kg significantly elevated the concentrations of DNA, RNA, and protein in duodenal mucosa (P<0.05); dietary SB at the level of 1000 mg/kg significantly increased the concentration of DNA, and protein

Table 4
Effect of dietary sodium butyrate on xylose absorption and morphology of the jejunal mucosa

	The level of sodium butyrate (mg/kg)				S.E.M.	Linear	Quadratic
	0	500	1000	2000			
Xylose absorption							
Concentration of xylose (mmol/l)[a]	4.81	3.98	5.93	5.07	0.264	NS	NS
Jejunal microscopic morphological items							
Villus height (mm)	0.79	0.86	0.85	0.87	0.007	NS	NS
Crypt depth (mm)	0.21	0.20	0.19	0.17	0.030	NS	NS
Villus height/crypt depth	3.96	4.35	4.56	5.24*	0.164	P<0.01	P<0.01

[a] Concentration of xylose of solution in the side of jejunal mucosa.
* Means were significantly different compared to the control (P<0.05).

Table 5
Effect of dietary sodium butyrate on the concentration of DNA, RNA, and protein in mucosa of duodenum and jejunum

	The level of sodium butyrate (mg/kg)				S.E.M.	Linear	Quadratic
	0	500	1000	2000			
Duodenal mucosa (mg/g)							
DNA	3.23	3.82*	4.01*	2.84	0.128	NS	<0.01
RNA	2.89	3.76*	2.91	2.59	0.138	NS	<0.05
Protein	34	41*	46*	35	1.3	NS	<0.01
Jejunal mucosa (mg/g)							
DNA	2.74	2.76	2.79	2.88	0.115	NS	NS
RNA	2.85	2.92	3.08	2.88	0.058	NS	NS
Protein	34	35	36	37	1.4	NS	NS

* Means were significantly different compared to the control (P<0.05).

in duodenal mucosa (P<0.05). Dietary SB did not influence the concentration of DNA, RNA, and protein in jejunal mucosa (P>0.05).

4. Discussion

Dietary SB had a positive effect of body weight gain from 0 to 21 days with the supplementation level of 500 and 2000 mg/kg, but a negative effect on FCR with the supplementation level of 2000 mg/kg. Thus, in the current study, the level of 500 mg/kg was the best for chickens. Dietary SB increased the average daily body mass gain and feed consumption, and reduced FCR (Galfi and Bokori, 1990). However, no significant effect on body weight gain was observed by oral administration of SB (Furuse et al., 1991) because the level of SB was too low. The results proved the beneficial effects of SB supplementation in chicken feed. In contrast to other organic acids, SB was used for chicken feed in much lower concentrations (0.5 g/kg) than citric acid, acetic acid and propionic acid (4–20 g/kg) (Rafacz-Livingson et al., 2005; Furuse et al., 1991; Izat et al., 1990) and could easily be mixed in the diet because SB was powdery.

The absence of any effect of SB on concentration of SCFAs in the intestine confirmed that organic acids such as SB were absorbed in the fore part of the intestine as shown in previous studies (Bolton and Dewar, 1965; Hume et al., 1993). Butyrate is absorbed through the following two ways (Cook and Sellin, 1998): Firstly, the diffusion of protonated SCFAs. The protons from the exchange of Na/H, K^+–H^+–ATPase and the metabolism of bacteria can acidify the colonic antrum, and lead to the increase of butyric acid which enters into the cells by diffusion. The pH in colonic antrum is slightly acidic compared to the systemic circulation, which induces diffusive movement of butyric acid. Secondly, anion exchange studies in membrane vesicles have produced evidence for a family of anion exchangers that mediate SCFAs:HCO_3^- exchange and entry across the apical membrane.

Results of effect of organic acid on gastrointestinal microflora are variable. However several reports have shown a decrease in coliform count and an increase in *Lactobacillus* count (Izat et al., 1990; Wielen et al., 2000). However in the current study, SB reduced the

number of *Lactobacillus* in jejunum. Knarreborg et al. (2002) also indicated that butyric acid inhibited the growth of lactic acid bacteria and coliform bacteria. The possible reason was due to the inhibition of bacteria of SB in the fore digestive tract (crop, gizzard and duodenum), especially bacteria producing lactic acid.

Results showed that dietary SB did not influence the absorption function of jejunum. The D-xylose test assessed intestinal absorption. D-Xylose was mainly absorbed in the duodenum and proximal jejunum and could not be metabolized by intestinal cells (Craig and Atkinson, 1988). Ford and Coates (1971) showed differences in glucose absorption between germ free and conventional birds with in vitro method, and Yokota and Coates (1982) showed no difference *in vivo*. *In vitro*, butyrate was an inhibitor of cell division whereas *in vivo*, butyrate was a stimulator of the gastrointestinal growth (Galfi and Neogrady, 2001). Therefore, the present result was needed to be confirmed by the further research *in vivo*.

The current experiment showed that SB had no influence on the villus height and crypt depth of hind jejunum, but increased the ratio of villus height to crypt depth. SB increased the length of ileal microvilli and the depth of caecal crypts (Galfi and Bokori, 1990). The ratio of villus height to crypt depth showed an effect because of decrease crypt depth (NS) and slightly higher villous height (NS); it can be seen as a better ratio for digestive tract maintenance. The reason might be the effect of other products produced by microflora on enterocytes (Bardocz et al., 1995).

In cecum-resected rats fed with a non-fibre diet and infused with 40 mmol/l butyrate or mixture of SCFAs in colonic antrum, the growth of colon significantly increased, which included the mucosal weight as well as the concentration of protein, DNA, and RNA (Friedel and Levine, 1992). In massive small-bowel-resected rats, SCFAs also significantly increased the weight of mucosa and the concentration of DNA, RNA, and protein (Koruda et al., 1988). In ruminants and non-ruminant herbivores, the metabolic roles of SCFAs were investigated in detail and known to stimulate epithelial cell division in the digestive tract (Sakata and Yajima, 1984). The weights of duodenum, jejunum and ileum were significantly increased by intraperitoneal administration of butyric acid, but no significant effect was observed by oral administration (Furuse et al., 1991). The reason might be insufficient oral administration of butyric acid. The present work showed that dietary SB increased the concentration of DNA, RNA, and protein in duodenal mucosa, but it did not influence the concentration of DNA, RNA, and protein in jejunal mucosa. The results suggested that SB stimulated the growth of duodenal mucosa. The difference between duodenal mucosa and jejunal mucosa might be due to the absorption of SB in the fore digestive tract before arriving to the jejunum.

In contrast to other organic acids in larger quantities and in acid form, the effect of SB could be mainly due to its extensive biological action, not its acidification because SB did not decrease the pH of the diet (unpublished observations) which was consistent with the result in pigs (Galfi and Bokori, 1990).

5. Conclusion

Dietary SB supplementation at the level of 500 mg/kg enhanced growth performance and stimulated the growth of duodenal mucosa in broilers. Dietary SB inhibited the growth

of lactobacillus in the jejunum. In order to exert the influence on the hind digestive tract, SB was recommended to be released slowly by embedding or esterification.

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