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Intestinal morphology and intestinal mucosal immune function as enhanced by the long-term feeding supplementation of sodium butyrate in adult breeder roosters

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Abstract
Butyrate is major short-chain fatty acids (SCFA), produced by anaerobic bacterial metabolism, and it showed beneficial health effects in several animal species such as poultry. This study aims to investigate the effects of sodium butyrate (SB) on morphological parameters and immune response gene expressions associated with the intestinal mucosa barrier functions in Chinese Xue Shan breeder roosters. Three hundred adult roosters at 22 weeks were randomly divided into 2 separate groups, control group (fed basal diets) and sodium butyrate group (fed basal diet sublimated by 0.05 % of sodium butyrate). Both groups were fed for 23 consecutive weeks with six replicates per group (each replicate consist of 25 birds). The results showed that the diet supplemented with 0.05% sodium butyrate was observed to maintain the body weight, and, significantly eleveted villus height in jejunum, ileum and villus height to crypt depth ratio in jejunum, as well as increasing the concentration of IL-6 (in the jejunum and ileum), TNF-α (in the ileum). Moreover, SB supplementation significantly up-regulated mRNA expression of mucin (MUC2) in ileum mucosa, while there is no significant increase in the mRNA expression of any of the claudin-1, occluding or zo1 in jejunum and ileum intestinal mucosa. Dietary SB supplementation significantly decreased the IL-β1 (in the serum) these results indicated that sodium butyrate maintains body weight and improve functions of the intestinal barrier led by the enhancement of its morphological development, regulating the immune response and up-regulating MUC2 gene expressions in ileum mucosa. Sodium butyrate at level 0.05 % had slight effects on the intestinal tight junction in adult breeder roosters. Further studies are needed to investigate the effect of the sodium butyrate and its interactions with other feed ingredients in poultry diets.
Keywords: Sodium butyrate, Immune function, Intestinal mucosa, Breeder Roosters.

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Introduction

The use of feed additives for nutritionally regulating the immune status might bring useful effects leading to improve poultry health and production. The poultry industry has become a significant economic activity in numerous countries. Under conditions of large-scale rearing facilities (modern system), poultry exposure to stress, disease-related problems and deterioration of environmental conditions often occur, this leads to severe economic losses. Intestinal health is main factor that was found to be affecting the performance of birds, thus, the economics of poultry production [1]. Likewise, the gut health development is considered crucial for birds' welfare and productivity. Also, Intestinal mucosal adaptability is key to survival as it contains a variety of biological effects, including nutrients absorption, barrier function, immune reservoir, and injury response.

Antibiotics have been used since the early 1950s in poultry feed so as to improve poultry health and production [2]. However, the current concerns of antibiotic residues and resistance caused the formulation of restrictions regarding antibiotics use in poultry [3, 4]. The use of antibiotics in poultry feed as growth promoters has banned been in the EU since 2006 [5, 6]. As a result, poultry nutritionists were compelled to look for new alternatives to overcome the adverse effects of antibiotics [7, 8]. Recently, butyrate produced from dietary fiber fermentation by bacteria is considered as a potential alternative to antibiotic growth promoter [9, 10]. Several studies demonstrated the beneficial effects of dietary supplementation of SB on the growth performance and intestinal integrity in swine and broiler chickens [11-14]. Sodium Butyrate is an important energy source for the intestinal epithelial cells [15] as well as for the reduction of the expression of pro-inflammatory cytokines in normally reared chicken [14]. Moreover, SB plays a vital role by stimulating small intestinal epithelium growth, cell proliferation and differentiation [16], as well as, promoting intestinal health [17]. Furthermore, butyrate may have an impact on oxidative stress [18] and inflammation [19]. Epithelial barrier is found to be vital for regular functioning of intestines and impairment may lead to inflammation. Numerous studies have shown that sodium butyrate is directly attributing in regulating intestinal barrier functions [18, 20-23]. Intestinal barrier function can be realized through regulating mucins and tight junction (TJ) proteins, which play a critical role in the maintenance of intestinal health and animal performance. A recent study has shown a novel role played by the sodium butyrate in the natural immunity by

inducing the host defense peptides (HDPs) and improving disease resistance [24]. The present experiment aims to explore the effect of long-term dietary supplementation of sodium butyrate on the intestinal morphology, intestinal mucosal immune function and tight junction in adult Chinese Xue Shan breeder roosters.

Materials and methods

Birds and experimental treatments

Sodium butyrates (98%) were obtained from Lihua Industrial Corporation (Changzhou, China). Experiments were carried out in Jiangsu Lihua Animal Husbandry Stock Co, Ltd. (Jiangsu, China) farm. A total of three hundred 22 weeks old, adult local Chinese breeder roosters (Xue Shan) that depicted similar body weights were randomly assigned into two dietary treatment groups, with six replicates, each cluster comprised 25 birds. The control group was provided with commercial feed formulated based on the NRC (1994) standards, while the experimental group fed with the same commercial feed supplemented with 0.05 % SB. Birds were placed in wired cages under controlled conditions with a photoperiod of 16 h light, 8 h dark at ambient temperature (about 21°C), and each bird was fed by 130g basal diet per day. The roosters were supplemented with sodium butyrate from 22 to 45 weeks of age, and the experiment was later continued for 23 weeks. Basal diet composition and nutritive value are listed in Table 1. The dosage of 500 mg/kg or 0.05% sodium butyrate was prepared based on Hu et al. (2007) who reported that sodium butyrate at the level of 500 mg/kg enhanced growth performance and intestinal mucosa in broiler chicken. [11].

Birds and tissue collection

Twelve birds from each treatment were randomly picked at end of experimental period from each replicate, weighed and murdered by cervical dislocation without previous starvation. Blood samples were collected and centrifuged immediately at 3500 rpm for 10 min at 4°C then the serum was stored at -20 °C until analysis. After decapitation, heart, liver, spleen, proventriculus and gizzard were cleaned with a physiologic saline solution, dried with filter paper, and weighed to observe the gross anatomical changes. While jejunum and ileum tissues were removed immediately from the middle part of each intestinal section and cut into fragments of approximately 5cm length. The intestinal segmentation was done according to the method of [25] where the jejunum was cut from the pancreatic loop to Meckel's diverticulum, and the ileum was cut from Meckel's diverticulum to the ileo-caeco-colic junction. Fragments were then opened, washed with 0.9 % NaCl, and immersed in 10 % buffered formaldehyde solution for 48 hours for histomorphometric study. Portions of the jejunum and ileum tissues were cleaned from residual food, immediately frozen in liquid nitrogen and then, stored at -80 °C until further use.

Table 1. Composition of the basal diet and nutritive value

Ingredients	Composition(%)
Corn	63.91
Soybean meal	9.6
Rice bran	23.57
Limestone	1.62
Fluid methionine	0.04
Choline chloride	0.13
Sodium chloride	0.3
Dicalcium phosphate	0.66
Vitamin premix[a]	0.05
Mineral premix[b]	0.12
Calculated nutritive value	
[1]ME (kcal/kg)	2,761
[2]CP (%)	12.98
Methionine (%)	0.28
Lys (%)	0.58
Calcium (%)	0.81
Total P (%)	0.80

a Provided per kilogram of diet: vitamin A, 6,000 IU; cholecalciferol, 2,500 IU; vitamin E, 40 mg; menadione,1.5 mg; thiamine, 3 mg; riboflavin, 12 mg; pyridoxine, 6 mg; vitamin B12, 35 µg; pantothenic acid, 10 mg; niacin, 60 mg; folic acid, 0.8 mg; biotin, 0.225 mg, b Provided per kilogram of diet: copper, 5.5 mg; iron, 70 mg; zinc, 55 mg; manganese, 60 mg; iodine, 0.4 mg; selenium, 0.15 mg, 1ME = metabolic energy, 2 CP = means crude protein.

Small intestinal morphology

For morphological analysis, segments of jejunum and ileum were gradually dehydrated with increasing alcohol concentrations (70% up to 100%), then, they were cleared in xylene, and subsequently embedded in paraffin wax. They were cut at a thickness of 6 µm (by a microtome), stained according to hematoxylin and eosin method (Shiva, Bernal et al. 2012). Each stained section was photographed using image processing and analysis system (XC10 Olympus microscope Tokyo- Japan) at 10 × magnification. The measurement of villus height was conducted from the tip of the villus to the crypt-villus junction, while the crypt depth was considered based on the depth of the invagination between adjacent villi. The measurements were performed on 5 villi and 5 crypts for each segment, and villus height to crypt depth ratio was calculated.

Jejunum and ileum mucosal cytokines

Each fragment of jejunum and ileum were opened longitudinally, and a clean glass slide was used to scrap off mucosa. Samples were transferred into sterile tubes (1.5 mL), and frozen immediately at -80◦C until analysis. Briefly, jejunum and ileum mucosa (0.5 g per sample) were weighed, diluted with 4.5 mL of 0.9 % salt solution, homogenized with a homogenizer (Wheaton, USA) and centrifuged at 3800 rpm for 15 min. The

supernatant was collected into a 1.5 mL sterile micro centrifuge tubes. Interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) concentrations were measured in serum, jejunal and ileal homogenates using ELISA kits (specific for chicken). Kits of the assays were obtained from the Nanjing Jiancheng Bioengineering Institute (China).

Table 2. Primers for mRNA analyses

Gene	Primer sequence	Accession number
MUC2	forward: 5′-CACCAACGGCAACTGAAATAGT-3 reverse:5′-GCCAAACCATGGGTAACTCACA-3′	NM_001318434.1
Claudin-1	forward: 5′- GGTGTACGACTCGCTGCTTA-3	NM_001013611.2
.	reverse:5′-CGAGCCACTCTGTTGCCATA-3′	
Occludin	Forward: 5′-CCGTAACCCCGAGTTGGAT-3′	NM_205128.1
	reverse:5′-ATTGAGGCGGTCGTTGATG-3′	
Zo-1	Forward5′- CTTCAGGTGTTTCTCTTCCTCCTC-3′	XM_413773
	Reverse5′ -CTGTGGTTTCATGGCTGGATC- 3′	
β –Actin	forward: 5′-ATCCGGACCCTCCATTGTC-3′	NM_205518
	reverse:5′-AGCCATGCCAATCTCGTCTT-3′	

Table 3. Body weights and organ relative weights of roosters treated with sodium butyrate supplementation

	Diets		
Item	Con	SB	p-value
Body weight (g)	1947 ± 112.30	1971 ± 51.21	0.84
Relative heart (%)	0.5 ± 0.08	0.55 ± 0.02	0.09
Relative liver (%)	1.46 ± 0.29	1.66 ± 0.23	0.09
Relative spleen (%)	0.10 ± 0.02	0.10 ± 0.03	0.63
Relative proventiculus (%)	0.21 ± 0.06	0.24 ± 0.03	0.19
Relative gizzard (%)	0.96 ± 0.29	1.05 ± 0.20	0.43

Con: Roosters fed a basal diet.
SB: Roosters fed a basal diet supplemented with 0.05 % sodium butyrate
Values are means ± standard error

Table 4. Small intestinal morphology of roosters fed with sodium butyrate supplementation

Item	Con	SB	p-value
Jejunum			
Villus height (μm)	893.8 ± 52.22	1154 ± 22.10*	0.01
Crypt depth (μm)	125.9 ± 8.70	109.7 ± 6.55	0.21
Villus height : crypt depth	7.19 ± 0.78	10.62 ± 0.86*	0.04
Ileum			
Villus height (μm)	450 ± 7.78	608.5 ± 14.28***	0.00
Crypt depth (μm)	108.5 ± 1.65	82.39 ± 4.50*	0.01
Villus height : crypt depth	5.61 ± 0.12	5.49 ± 0.25	0.71

Con: Roosters fed a basal diet; SB: Roosters fed a basal diet supplemented with 0.05% sodium butyrate; Means were significantly different compared to the control (P<0.05).

Table 5. Effects of dietary supplementation sodium butyrate on cytokine level in serum and intestinal mucosa in breeder roosters

Item	Con	SB	p-value
Serum			
IL-1β (ng/L)	14.80 ± 1.01	11.74 ± 0.36*	0.01
IL-6 (ng/L)	14.40 ± 0.81	12.08 ± 1.50	0.24
TNF-α (ng/L)	12.50 ± 0.97	10.95 ± 0.35	0.14
Intestinal mucosa			
Jejunum			
IL-1β (ng/L)	19.54 ± 0.82	18.19 ± 0.73	0.24
IL-6 (ng/L)	21.06 ± 0.47	24.31 ± 0.84**	0.00
TNF-α (ng/L)	16.92 ± 0.37	16.18 ± 0.53	0.27
Ileum			
IL-1β (ng/L)	20.76 ± 1.44	23.76 ± 1.02	0.11
IL-6 (ng/L)	22.93 ± 0.64	27.30 ± 1.02**	0.00
TNF-α (ng/L)	16.88 ± 0.79	20.05 ± 0.72*	0.01

Con: Roosters fed a basal diet; SB: Roosters fed a basal diet supplemented with 0.05% sodium butyrate; Means were significantly different compared to the control ($P<0.05$)

Gene expression of jejunum and ileum mucosa by qReal-Time PCR

Total RNA was isolated from the jejunum and ileum mucosa samples (6 samples per treatment) using TRIzol reagent (Invitrogen) according to the manufacturer's procedures. The quantity and purity of RNA were measured at 260/280 nm using the ND 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Reverse transcription to cDNA was performed using Reverse Transcription kit (Omniscript® TaKaRa Biotechnology Co. Ltd., Dalian, China). Real-time PCR was performed using a Bio-rad detection system 210 (Bio-rad IQ5 multicolor, USA) with SYBR Premix Ex Taq™ kit was obtained from Takara (Dalian, China). Rooster's muc2, occluding, claudin-1, zo-1, and β-actin from jejunum and ileum were amplified, and the primer sequences used for real-time PCR are listed in Table 2. PCR was performed under the following conditions: hold stage step of 95 °C for 30 sec, followed by 40 cycles of 95 °C for 5 sec, 60 °C for 30 sec; melt curve stage included 95 °C for 15 sec, 60 °C for 60 sec, 95 °C for 15 sec. Relative mRNA expression levels were expressed as $2^{-\Delta\Delta Ct}$ (ΔCt = Ct genes – Ct β-actin) and analyzed by the comparative cycle threshold (CT) method [26].

Statistical analysis

Experimental data was analyzed using the statistics software (Prism 5.0 statistical software; GraphPad Software, Inc., San Diego, CA). All data were analyzed following an independent-samples t-test, and results were presented as the mean ± standard error (SE). Significant differences were considered when the p-value was less than 0.05.

Results

Body and organ weight

All roosters were alive and healthy till the end of experiment. As shown in Table 3, the body weight was increased but not significant at (P<0.05) between the control group (1947 ± 112.3) and the SB group (1971 ± 51.2). Likewise SB supplementation diet did not significantly increase the relative organ weights until the end of the feeding trial.

Jejunum and ileum microscopic morphological structure analysis

Hematoxylin and eosin (H&E) staining of jejunum and ileum of birds fed with butyrate-supplemented diet at 45 weeks of age were presented in (Fig 1).

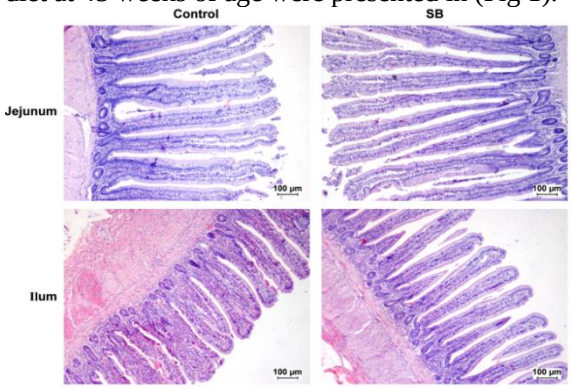

Fig 1. A representative area of crypt depth in normal and treated samples. In both jejunum and ileum. Haematoxylin and eosin (H&E) stained at 10X magnification.

Dietary sodium butyrate supplementation significantly (P < 0.05) increased the villus height in the jejunum and ileum at. Roosters fed with SB supplemented diet had lower (P < 0.05) ileum crypt depth compared to control birds and villus height to crypt depth ratio in the jejunum treatment group was increased significantly compared to the control group (P < 0.05), while no differences were observed between treatments and control groups in jejunum crypt depth and ileum villus height to crypt depth ratio Table 4.

Effect of sodium butyrate on cytokine levels in serum and intestinal mucosa (jejunum and ileum)

IL-1β serum levels were decreased significantly (P < 0.05) in treated birds compared to control birds Table 5, while no different change was detected in IL-6 and TNF-α concentration. While in dietary sodium butyrate supplementation significantly increased the IL-6 level in the jejunum and ileum mucosa (P < 0.05). Moreover, Birds in SB treatment exhibited higher (P < 0.05) ileum mucosa TNF-α concentration than the control group. There was no significant difference detected in the IL-1β concentration in jejunum and ileum mucosa between treated and control birds.

Relative mRNA Expression of MUC2, Occludin, Claudin-1, Zo-1 in jejunum and ileum mucosa

As shown in (Fig 2A), Dietary Sodium butyrate supplementation significantly (P<0.05) increased the MUC2 mRNA expression in birds ileum, although no significant effect in the jejunum was observed. While in both jejunum and ileum the sodium butyrate increase but not significant the mRNA expression levels of Occludin, Claudin-1, Zo-1 in treated birds compared to control birds (Fig 2 B, C, and D).

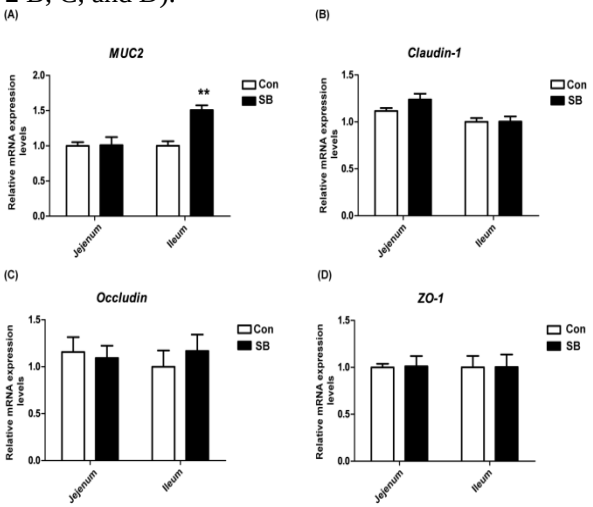

Fig 2. Effects of sodium butyrate on the relative mRNA expression of jejunum and ileum mucosal mucin2 and tight junction proteins of breeder roosters. Quantitative real-time PCR analysis of MUC2, occludin (OCLN) claudin -1 and zonula occludens protein-1 (ZO-1). **P < 0.01. Compare with control roosters

Discussion

In The intestinal mucosal structure reflects intestinal health. The function and structure of intestinal mucosa might be depending on the balance between several biological processes (cell proliferation, migration, and apoptosis) [27]. Therefore, Long villi and short crypts are usually considered to be a health index and function of the small intestine. Villus height and crypt depth were closely related intestinal absorptive capacity [28]. Furthermore, a greater proportion of the villus: crypt reflects high digestion and absorption capacity [29]. In broiler chicken, previous Studies showed that the villus height of the jejunum and ileum increased in response to sodium butyrate (fat coating), while Crypt depth and villus height to crypt depth ratio were unaffected [30]. Czerwinski

et al (2012) reported that jejunum villus height was significanlty increased in broiler received a diet intake that includes 300 mg/kg sodium butyrate [9], while Lu et al. (2008) reported that supplementation at a dose of 500 mg/kg of sodium butyrate increased the villus height and the villus height to crypt depth ratio in the jejunum [31]. In the present study, roosters fed dietary supplemented with (0.05%) sodium butyrate showed a significant improvement in intestinal structure and morphology. Additionally, the jejunum and ileum villus height were significantly increased. Birds in SB treatment had lower ($P < 0.05$) ileum crypt depth and higher villus height to crypt depth ratio in the jejunum compared with control birds. Although an increase in jejunum and ileum villus height was observed in birds group fed with SB-supplemented diet, this was not reflected in body and organ weights. This might be due to restricted feed consumption in this experiment. Thus, sodium butyrate might play a vital role in body weight maintains by prevented decreased the body weight by immune stress these results agree with a previous observation of Zhang et al. (2011), Who reported that supplemented diet with sodium butyrate remarkably protected the reduction in body weight gain affected by immune stress [14].

Cytokines is a class of low-molecular-weight proteins with various biological activities and play an important role in the regulation of immune and inflammatory responses. Butyrate promotes gut health [17] and affects intestinal inflammation [18] through the strength of the barrier function and positively affects the immune system [32, 33]. In numerous studies, short chain fatty acid (SCFAs) including butyrate have been shown modify the production of cytokines by human intestinal epithelial cells [34, 35]. Also, in mice Kim et al. (2013), reported that, short chain fatty acid (SCFAs) including butyrate increase the production of cytokines by intestinal epithelial cells [36]. Similarly, an In vitro study using dietary supplementation with sodium butyrate demonstrated a clear modulation of t- cell function in rat [37]. Although, little information has been available about the signaling pathways showing the use of sodium butyrate to alter the cytokine production, also little information is known about cytokine roles under normal physiological conditions. Interleukin-6 (IL-6) is associated with T- and B-cell growth and differentiation, Tumor necrosis factor-α (TNF- α) is responsible for the activation of local inflammation, Interleukin-1β (IL-1β) involved in a variety of cellular activities. Butyrate can produce bacteria Clostridium butyricum (C. butyricum) and this species of bacteria could induce sensitization by increasing pro-inflammatory cytokines and afford positive effects to the host by synthesizing the immunosuppressive cytokines [38-40]. In addition to, sodium butyrate supplementation could decrease of serum cytokines concentration in normal reared birds [14]. Results in the present study showed that the concentration of IL-1β in the serum was significantly decreased in the SB group compared with the control group. While the level of IL-6 was significantly increased in the jejunum and ileum mucosa in SB groups compared with the control groups. Moreover, TNF level in the ileum was considerably increased in the treatment group. These results may indicate that dietary supplementation of sodium butyrate influenced the immune response in Breeder roosters. Tight junction (TJ) and mucus layer formed two important structures to maintain the integrity of the gut. Tight junction (TJ) forms a seal between cells and epithelial tissue, while mucus layer covers the luminal surface of epithelial cells [41, 42]. Claudin and Occludin family are the most important components involved in regulating the physical barrier function in intestinal epithelia. Peng et al. [22] reported that butyric acid could improve the integrity of intestinal epithelium by increased the expression of tight junction protein. Besides that, sodium butyrate plays an important role in the intestinal tract, which has a positive effect on the recovery of tight junctions of the intestine and maintaining the integrity [24]. An In vitro study by Ma et al. [43], showed that, sodium butyrate could significantly increase the expression of ZO-1 and OCLN in Porcine small intestinal epithelial cells. Our results referred that dietary 500 mg/kg sodium butyrate might cause an improve in the expression levels of Occlodin, claudin, and zo-1 in both jejunum and ileum mucosa but the levels of expression between two diets was not significant at ($P< 0.05$). Lack of significant may be attributed to the low level of sodium butyrate supplemented in the diet. Mucin 2 is the main component of the intestinal gel mucus secretion serves as a barrier to protect the intestinal epithelium [44]. It was previously reported that butyrate could affect the barrier function by increasing mucin glycoproteins formation, which may improve protection against luminal agents [45]. In the current study, we observed that the relative expression of MUC2 mRNA was significantly higher in the ileum mucosa of sodium butyrate group than the control group. This result in agreement with previous studies done by Barcelo, et al. (2000) and Gaudier, et al. (2004) who stated that sodium butyrate could increase expression of MUC2 gene and the effect is dose dependent [46, 47]. Moreover, Butyrate increased colonic mucosal barrier by stimulating

the formation of mucin glycoproteins [48] and promote the synthesis of MUC2 [49, 50].

Conclusions

In conclusion, results of the 23 week-long feeding received 0.05% sodium butyrate supplementation in the diet can stimulate immune responses and improving intestine morphology, enhance the barrier functions of intestinal epithelium through the up-regulation of MUC2 expression. Our results suggest that incorporation of sodium butyrate in poultry diets has exhibits potential for healthier birds, Likewise, sodium butyrate may be used as an antibiotic alternative for animal production. From this result, we suggested higher oral doses of sodium butyrate might be required to protect early when birds are restricted feeding.

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