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Research Article

Assessment of Tributyrin As A Replacement for Antibiotic Growth Promoters in Broiler Diets: Effects on Performance, Selected Bacterial Population in Digesta, Intestinal Histo-Morphology and Immune Responses (Measured Through Vaccine Titres)

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Abstract

In a feeding trial of 42-days a flock of 800 male Cobb broiler chickens were supplemented with either an antibiotic growth promoter (AGP) or tributyrin (TB) in a 2 x 2 factorial design where two levels of AGP (0 and 500 mg/kg diet) and two levels of TB (0 and 500 mg/kg diet in starter, 250 mg/kg in grower and finisher) were used as the factors. The negative control (NC) diet was devoid of either of these treatments. Body weight, feed intake and feed conversion ratio were refractory to the effects of AGP, TB and their combinations ($p > 0.05$). Supplementation of the AGP and TB, either alone or together, decreased the weight of the small intestine relative to the body weight as compared to the control group ($p < 0.05$). Under the influence of the TB height of the villi increased in the jejunum (main effect TB $p < 0.001$, AGP x TB interaction $p > 0.05$). Supplementation of AGP and TB decreased numbers of *Salmonella* and *Escherichia coli* in the pooled digesta collected from the small intestine and TB, either alone or in combination with AGP, was more efficient in this regard (main effect AGP $p < 0.05$, main effect TB $p < 0.01$, AGP x TB interaction $p < 0.05$). Supplementation of TB alone increased the count of *Lactobacillus* (main effect TB, $p < 0.05$) but TB and AGP together decreased the same (TB x AGP interaction $p < 0.05$). Both AGP and TB decreased the number of *Clostridium perfringens* ($p < 0.05$) as compared with the NC group. The data revealed that the TB was not as efficient as the AGP in reducing the number of *C. perfringens*, the count of which was higher in the former group ($p < 0.05$). The present study indicated that under standard management condition, where the birds are not exposed to any stress per se, TB may be as good as an in-feed AGP in sustaining broiler performance and the effects are achieved mostly by maintaining a healthy intestinal milieu with a greater number of beneficial commensal organisms and perhaps a superior absorptive surface. Hence, TB may constitute an effective replacement strategy for antibiotics in the feeding regimens of broiler chickens.

Keywords: Tributyrin; Broiler Chickens; Intestinal Histo-Morphology; Immune Responses

Abbreviations

BW: Body Weight; ADFI: Average Daily Feed Intake; ADG: Average Daily Gain; AGP: Antibiotic Growth Promoter; BA: Butyric Acid; BMD: Bacitracin Methylene Disalicylate; FCR: Feed Conversion Ratio; IBD: Infectious Bursal Disease; ND: Newcastle Disease; SCFA: Short Chain Fatty Acids

Introduction

Short-chain fatty acids (SCFA) have been widely used as feed additives in poultry for the control of pathogenic bacteria [1]. Butyric acid is considered to be one of the most important SCFA, which is critical for establishment and maintenance of the intestinal health. Intestinal cells, particularly those in the colon, take up the butyric acid where it is used for the production and maintenance of colonic homeostasis [2] though for this the butyric acid must escape degradation and absorption in and from the upper small intestine. The role of butyric acid as a source of energy for the epithelial cells, sodium and water absorption, proliferation and differentiation of epithelial cells, villi development and improvement in gut defence system has been established [3,4]. Generally, the antimicrobial activity of short-chain fatty acids against pathogenic bacteria seems dependent on the type of fatty acid, form, pH, exposure time, degree of sensitivity of specific types of pathogens, and quantity used [5-7]. It has been shown that fatty acids and their monoglycerides are more effective in inhibiting bacterial growth [8,9] than are di- and triglycerides of these same fatty acids [10].

Generally, the antimicrobial activity of organic acids is attributed to their ability to pass through the cell membrane and dissociate in the more alkaline cell thereby acidifying the cell cytoplasm

(Kashket, 1987; Salsali., *et al.* 2008). Butyric acid, being a SCFA with 4 carbon atoms, has been shown to reduce *Salmonella* colonization in the ceca [11] and invasion of *Salmonella* bacteria in the chicken cecal epithelial cells [12]. Also, it has been reported that supplementation of butyric acid glycerides in diets of broiler chickens increased their carcass weight and breast meat [13]. Monoglycerides of SCFA have comparable or better antimicrobial activity compared with the free fatty acids [14-16]. Monoglycerides of fatty acids have an advantage in handing because they do not possess the stringent smell associated with the free acids and are released only under the influence of lipase in the small intestine [1].

With a comprehensive ban looming over the usage of in-feed antibiotics as growth promoters in animals being used in human food chain it has become imperative for the poultry producers to get a suitable replacement which can provide protection to the birds against the possible insults to their guts. Short chain fatty acids like acetate and propionate are used successfully used as water sanitizers in poultry production and *in vitro* SCFA like butyrate and valerate have been found to be highly efficacious against gram negative bacteria like *Salmonella enterica* [17]. Hypothetically this effect should be obtained when birds are supplemented with glycerides of butyric acid and the effects should get accentuated by the effects of the glyceride moiety on development of the villi in the small intestine.

With the above background and hypothesis the present study was designed where broiler chickens were fed with diets supplemented with a glyceryl ester of butyric acid either alone or in combination with a common in-feed-antibiotic growth promoter (AGP) to ascertain if the butyric acid ester, either singly or together with the AGP could bring about improvements in terms of performance of the experimental broilers. The further objectives were to asses the effects of the butyric acid ester on the mucosal architecture and microbial population of the small intestine and finally to assess if the butyric acid ester could be used as an effective replacement for the AGPs in the feeding regimen of broiler chickens.

Materials and methods

Dietary treatments

The experimental design was a completely randomized design in which the experimental birds were distributed into 4 dietary groups and were fed with a basal diet devoid of any gut acting growth promoter or the same supplemented with either an antibiotic growth promoter (AGP) containing 10 mg/kg of bacitracin methylene disalicylate, or a product containing tributyrin consisting of uncoated glyceryl ester of butyric acid (TB, Prophorce-SR-130, Perstorp Chemicals, Sweden) or both. The level of inclusion of the AGP was 500 mg/kg diet and that of TB was 500 mg/kg in starter and 250 mg/kg in grower and finisher diets. Thus, the dietary treatments were, (i) Control, (ii) Control + AGP, (iii) Control + TB and (iv) Control + AGP + TB. The treatments, TB and the AGP, were mixed along with the other additives as mentioned in Table 1, with a fixed quantity of ground corn to form a premix and it was this premix which was added as a whole with the raw materials which were mixed together after grinding in a paddle type mixer with a capacity of 100 kg. It should be noted here that butyric acid derivatives are highly costly and does not always seem feasible in broiler formulations when one looks at it from a commercial point of view. The basis of the dose selection was hence aimed at selecting the lowest possible inclusion levels in diet which could yield the maximum benefit with the least cost involvement. Thus, contrary to the comments made by Leeson., *et al.* [13] regarding the ideal inclusion level of butyric acid derivatives to yield discernible performance benefits in poultry a much lower inclusion level was used in this study. It should further be noted here that the primary objective of the study was to ascertain the efficacy of butyric acid ester as a performance enhancer at these dose levels tested and not to analyse the cost benefit per se. Hence, the discussion on the economics of the dietary treatments was kept out of the purview of this paper.

General bird husbandry and measurement of performance traits

The experiment was conducted with a flock of 800 Cobb broilers (initial mean body weight 46 g) which were put in test for a period of 42 days. The chicks were placed in pens on litter and there were 8 replicate pens in each treatment groups. Each pen consisted of 25 chicks and there were 200 chicks in a single dietary group. Distribution of the chicks between the treatments and between the pens within a treatment was done following a completely randomized block design to minimize pen effects. Diet and drinking water were offered ad libitum. The birds were fed with a starter (1-14 d and a grower (15-28 d) crumble and a finisher (29-42 d) pellet, the ingredients and chemical composition of which are given in Table 1. Raw materials of same lot were used for preparation of the diets and diets of all stages were prepared afresh just prior to the start of the respective feeding stage. The birds were vaccinated against Marek's disease at the hatchery and then against Newcastle disease (ND) on 5 and 12 d of age and infectious bursal disease (IBD) on 20 d of age. The lighting schedule involved 24 h light during the first week and 20 h of light after that.

Ingredients	T1 – Control			T2 – AGP[1]			T3 – TB ester[2]			T4 – AGP+TB ester		
	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter	Grower	Finisher	Starter	Grower	Finisher
Maize	525	579.3	604	525	579.3	604	525	579.3	604	524	578.55	603.25
Soybean meal 45% CP	400	345	302.9	400	345	302.9	400	345	302.9	400	345	302.9
De-oiled rice bran	0	0	0	0	0	0	0	0	0	0	0	0
Vegetable oil	36	40	60	36	40	60	36	40	60	36	40	60
Di-calcium phosphate	16.5	14.5	12.5	16.5	14.5	12.5	16.5	14.5	12.5	16.5	14.5	12.5
Limestone powder	8.7	8.7	8.7	8.2	8.2	8.2	8.2	8.45	8.45	8.7	8.7	8.7
Common salt	3.5	2.5	2	3.5	2.5	2	3.5	2.5	2	3.5	2.5	2
Sodium bi carbonate	2	2	2	2	2	2	2	2	2	2	2	2
L-lysine HCl	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
DL-Methionine	2.7	2.6	2.5	2.7	2.6	2.5	2.7	2.6	2.5	2.7	2.6	2.5
L-Threonine	0.7	0.6	0.5	0.7	0.6	0.5	0.7	0.6	0.5	0.7	0.6	0.5
Toxin binder	1	1	1	1	1	1	1	1	1	1	1	1
Trace mineral premix[3]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin premix[4]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride 60%	0.7	0.6	0.7	0.7	0.6	0.7	0.7	0.6	0.7	0.7	0.6	0.7
Coccidiostat (Salinomycin)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
AGP	-	-	-	0.5	0.5	0.5	-	-	-	0.5	0.5	0.5
TB ester	-	-	-	-	-	-	0.5	0.25	0.25	0.5	0.25	0.25
Antioxidant	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Phytase 5000[5]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
NSPase Enzyme	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table 1: Composition of the experimental diets (g/kg unless stated otherwise).

[1] Bacitracin methylene disalicylate (10%); [2] Glyceryl ester of butyric acid (Prophorce SR 130, Perstorp Holding AB, Neptunigaten, Sweeden); [3] Contains (mg/kg) copper (5), iron (15), manganese (30), zinc (25), selenium (0.25), iodine (2) and chromium (0.25) as yeast protein chelate; [4] Contained (per kg premix) retinyl acetate 3.75 mg, 1,25-hydroxy-cholecalciferol 4 mg, DL-a-tochopheryl acetate 30 mg, menadione 4 mg, thiamine propyldisulphide 3 mg, riboflavin tetrabutyrate 8 mg, riboflavin tetrabutyrate 8 mg, methylcobalamin 0.025 mg, sodium pantothenate 15 mg, pyridoxine 5 mg, niacin 60 mg, biotin 0.2 mg and folic acid 2 mg; [5]Modified *Escherichia coli* phytase (Quantum Blue, AB Vista Feed Ingredients, Marlborough, UK) having a declared phytase activity of 5000 U/g.

Nutrient	Starter	Grower	Finisher
Metabolizable energy MJ/kg	12.1	12.6	13.2
Crude Protein	220	200	180
Standardized ileal digestible amino acid			
Lysine	12.2	11	10.0
Methionine	4.5	4.18	4.00
Met + Cys	8.54	8.03	7.60
Threonine	7.81	7.04	6.8
Tryptophan	2	1.9	1.8
Arginine	12.8	11.9	11
Isoleucine	7.93	7.37	6.9
Valine	9.39	8.58	7.9
Calcium	9	8	7.5
Available P	4.5	4	3.6
Sodium	2.4	2	1.8
Choline mg	1800	1600	1600

Table 2: Calculated chemical composition of the basal diet g/kg unless stated otherwise.

Diets were formulated on ideal amino acid ratio (the values indicate the standardized ileal digestible amino acid content of the diets based on the reports published by Evonik India on Indian raw materials and their amino acid contents, 2015).

Body weight and feed intake was recorded pen wise at 14, 28 and 42 d of age. Feed was offered daily at 0800 h and the amount left was quantified after 24 h. Average daily body weight gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) was calculated pen wise for the periods of 1-14 d, 15-28 d, 29-42 d and 1-42 d. The flock was checked every day for mortality and the cumulative liveability was calculated at the end of the trial.

Measurement of empty gut weight

The weight of the empty gut (from the proximal duodenum to the terminal ileum excluding the caeca) was recorded with the hypothesis that dietary supplementation of both AGP and BA would reduce the total bacterial load in the small intestine and as a result the inflammatory process induced by colonization of mainly the pathogens would be lessened and hence the small intestine should be thinner and lighter. For this, at the end of the trial (42 d) 8 male birds having body weight close to the mean body weight of the group were selected from each of the treatment groups (1 bird per pen) and they were kept fasted for 2-3 hours. Following recording of body weight, the birds were stunned manually and killed by exsanguination. The viscera were opened and the small intestine including the ceca was severed out. The small intestine was emptied of the residual digesta by applying gentle pressure which was enough to expel all the remaining digesta present inside, washed by phosphate buffer solution to remove the tissue debris and soaked in tissue papers before the weight was recorded. It should be noted here that since the birds were kept off fed for some time before being slaughtered, the quantity of the residual digesta was quite nominal and could be expelled by application of pressure only. The weight of the empty small intestine was expressed as g/100 g of live weight.

Enumeration of bacteria in pooled digesta samples

At 42-d one bird from each pen was selected randomly (8 birds from each dietary group). The birds were mechanically stunned and killed by exsanguination and the small intestine was removed aseptically. The part of the small intestine from the Meckel's diverticulum to the caeca was ligated with twines and was severed out with the contents inside and stored at 4oC. Within 48 h, the intestinal contents were collected in sterile polystyrene tubes by applying gentle pressure with a spatula. Enumeration of the specific bacteria was performed according to the methodology described by Haldar., *et al.* [18]. Approximately 1 g digesta sample was homogenized in a tissue grinder with double the volume of ice cold phosphate buffered saline. The homogenized samples were decimally diluted, and 1 ml of the diluted sample was cultured aerobically on specific agar plates for enumeration of total *Salmonella Escherichia coli* and *Lactobacillus* spp. For enumeration of *Salmonella* a peptone, yeast extract and bile salt base agar were used while for *E. coli* a tryptone, peptone and bile salt based agar was employed; for estimation of *Lactobacillus* (all from Hi-Media Laboratories, Mumbai, India) were used. *Clostridium perfringens* was cultured anaerobically in reinforced clostridial agar (M 154, Hi-Media Laboratories, Mumbai, India) for 48 h in presence of carbon di oxide and all visible colonies were enumerated in a colony counter.

Histology of the small intestine

The histological study of the small intestine (SI) was performed to evaluate the effects of the TB additives on the histomorphology and integrity of gut. At 35 d one bird was randomly selected from each of the cages (8 chickens per treatment) and euthanized by cervical dislocation and bleeding of the carotid artery. The SI was removed and washed with sterile phosphate buffered saline (PBS) and the contents were emptied into sterile plastic containers which were stored at 4oC for microbiological assay to be described later. Segments measuring 2-cm in length from the mid-points of the jejunum were cut and fixed in 10% buffered formalin. The tissue samples were later embedded in paraffin, and a 2-µm section of each sample was placed on a glass slide and stained with hematoxylin and eosin. Histological sections were examined with a phase contrast microscope coupled with an integrated digital imaging analysis system. The variables measured were villus height, crypt depth and thickness of the lamina propria, tunica muscularis and tunica serosa. Villus height was measured from the tip of the villus to the top of the lamina propria, and the crypt depth was measured from the base up to the region of transition between the crypt and villus. Ten measurements were taken per bird for each variable the average of these values was used for statistical analysis [18,19].

Assessment of humoral immune response

The humoral immune responses against ND and IBD post vaccination against these diseases were determined by haemagglutination inhibition (HI) test (OIE, 2010) and an enzyme linked immunoserbent assay of IBD antibody respectively. The humoral immune responses against ND and IBD were considered as a model in this study to predict the immune modulation effects of the TB esters. Blood samples were collected at 12 and 30-d of age from the brachial vein of the chicks in polystyrene tubes. Immediately after collection the tubes were placed in ice for 60 min to clot the blood. The serum was separated from the cells by centrifugation at 2500 × g for 10 minutes and the serum thus harvested was stored at −20°C. For ND the results were validated against a negative control serum with titer value less than 1/4 and a positive control serum. The titers were expressed as log2 and the values were pooled group wise for statistical analysis.

Statistical analysis

The data related to body weight, ADFI and FCR was pooled pen wise while the data related to weight of the small intestine, intestinal microbiology, histo-morphology of the small intestine and immunity of the birds a single bird was considered as an experimental unit. The data were analyzed in a 2 x 2 factorial design in the general linear model of SPSS (version 17.0) where two levels of supplemental BMD (0 and 500 g/t) and two levels of supplemental ProPhorce SR 130 (0 and 500/250 g/t) were used as the main factors. All values were expressed as mean and pooled standard error

of mean. Probability values at P < 0.05 were considered as statistically significant while those at P < 0.1 were described as trends.

Result

Body weight, ADG and ADFI data are presented in Table 3. Body weight at 42-d was similar across the groups (p > 0.05). At the given points of measurements neither AGP nor TB supplementation elicited any effect on body weight and ADG of the birds (main effect AGP/TB p > 0.05). Average daily feed intake too was not affected by dietary supplementation of AGP and TB either alone or in combination (p > 0.05). Total feed intake was numerically higher in the T1 (control) group as compared with the T2 (AGP), T3 (TB) and T4 (AGP+TB) groups. Feed conversion ratio (Table 4) during 1 to 14-d was superior in the T2, T3 and T4 groups supplemented with AGP or TB either alone or in combination as compared with that in the T1 group (main effect AGP p < 0.001, main effect TB, p < 0.05, AGP x TB interaction p < 0.05). However, a similar difference was not discernible at the subsequent points of measurement (p > 0.05).

	14-d	28-d	42-d	1 to 14-d	14 to 28-d	1 to 28-d	29 to 42-d	15 to 42-d	1 to 42-d
	Body weight g			Average daily gain in live weight g					
T1-Control[1]	488.5	1486.8	2680.70	31.6	71.3	51.5	85.3	78.3	62.7
T2-AGP[2]	510.7	1479.0	2673.06	33.2	69.2	51.2	85.3	77.2	62.5
T3-TB [3]	501.3	1476.3	2692.85	32.5	69.6	51.1	86.9	78.3	63.0
T4-AGP+TB [4]	497.7	1463.7	2682.51	32.3	69.0	50.6	87.1	78.0	62.8
Pooled SEM	3.30	13.42	14.38	0.24	0.59	0.28	0.82	0.50	0.34
Main effect AGP *P*	0.15	0.54	0.77	0.12	0.52	0.79	0.80	0.87	0.97
Main effect TB ester *P*	0.98	0.43	0.72	0.15	0.26	0.53	0.96	0.53	0.77
AGP x TB ester interaction *P*	0.05	0.89	0.96	0.05	0.53	0.89	0.97	0.69	0.97
	Feed intake g			Average daily feed intake g					
T1-Control[1]	548.0	2094.2	4461.4	39.1	110.4	74.8	147.9	129.2	99.2
T2-AGP[2]	553.3	2069.9	4393.3	39.5	108.3	73.9	166.0	137.1	104.6
T3-TB [3]	549.3	2045.2	4398.5	39.2	106.9	73.0	168.1	137.5	104.7
T4-AGP+TB [4]	535.9	2090.5	4424.2	38.3	111.0	74.7	166.7	138.9	105.3
Pooled SEM	3.25	12.98	78.91	0.23	0.96	0.46	5.49	2.79	1.88
Main effect AGP *P*	0.53	0.69	0.44	0.52	0.59	0.69	0.46	0.42	0.44
Main effect TB ester *P*	0.22	0.59	0.42	0.21	0.82	0.59	0.36	0.39	0.42
AGP x TB ester *P*	0.16	0.20	0.54	0.16	0.11	0.20	0.39	0.57	0.54

Table 3: Live weight and live weight changes and feed intake in experimental birds (means of 8 pens per group, n = 25 birds per pen).

[1]Control diet without any added growth promoter; [2]Control diet supplemented with bacitracin methylene di salicylate 10%; [3]Control diet supplemented with a glyceryl ester of butyric acid (Prophorce SR 130, Perstorp Holdings, Sweeden); [4] Control diet supplemented with both AGP and TB ester.

Dietary groups	Feed conversion ratio			Liveability %	Production efficiency
	1-14 d	1-28 d	1-42 d		
T1-Control[1]	1.239[b]	1.454	1.693	94.0	354.7
T2-AGP[2]	1.191[a]	1.445	1.673	95.5	365.4
T3-TB [3]	1.207[b]	1.430	1.662	96.5	372.1
T4-AGP+TB [4]	1.187[a]	1.475	1.679	92.5	353.7
Pooled SEM	0.007	0.008	0.029	0.96	3.54
Main effect AGP *P*	<0.001	0.23	0.37	0.52	0.52
Main effect TB ester *P*	0.03	0.86	0.47	0.9	0.71
AGP x BA ester *P*	0.04	0.08	0.54	0.17	0.21

Table 4: Feed conversion ratio and liveability of experimental broilers (means of 8 pens per group, n = 25 birds per pen).

[1]Control diet without any added growth promoter; [2] control diet supplemented with bacitracin methylene di salicylate 10%; [3] control diet supplemented with a glyceryl ester of butyric acid (Prophorce SR 130, Perstorp Holdings, Sweeden); [4] control diet supplemented with both AGP and TB ester. Means in a column bearing dissimilar superscripts vary significantly.

Dietary supplementation of TB decreased the relative weight of the small intestine (Table 5) in the T3 and T4 groups as compared with that in the T1 group (main effect TB $p < 0.001$). Supplementation of AGP tended to decrease the relative weight of the small intestine (main effect AGP $p < 0.01$) in the T2 compared to that in the T1 group and there was an AGP x TB interaction as well ($p < 0.05$). On visual observation, the small intestine of the birds in the T3 and T4 groups receiving TB supplementation either alone or in combination with the AGP respectively had better tonicity as compared with the small intestine of the birds in the T1 and the T2 groups.

Dietary supplementation of AGP and TB decreased numbers of *Salmonella* and *E. coli* in digesta (Table 5) and TB either alone or in combination with AGP was found to be more efficient than AGP alone in this regard (main effect AGP $p < 0.05$, main effect TB $p < 0.01$, AGP x TB $p < 0.05$). Numbers of *Lactobacillus* spp. decreased in the T2 group due to dietary supplementation of AGP (main effect AGP $p < 0.001$) while TB supplementation in T3 diet increased the same as compared with that in the T1 group (main effect TB $p < 0.001$). In the T4 group receiving both AGP and TB *Lactobacillus* number was lower than that in the T3 group (AGP x TB $p < 0.05$).

Dietary groups	Small intestine	*Salmonella*	*E. coli*	*Lactobacillus*	*C. perfringens*
	% BW	CFU	CFU	CFU	CFU
T1-Control[1]	4.34[b]	6.80[c]	9.05[c]	7.85[b]	66.25[c]
T2-AGP[2]	3.81[ab]	6.53[b]	8.86[b]	6.79[a]	12.93[a]
T3-TB [3]	3.33[a]	6.31[a]	7.49[a]	8.60[d]	20.43[b]
T4-AGP+TB [4]	3.39[a]	6.21[a]	7.51[a]	8.24[c]	11.87[a]
Pooled SEM	0.10	0.04	0.13	0.12	4.12
Main effect AGP *P*	0.08	0.001	0.02	0.001	0.001
Main effect TB ester *P*	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
AGP x TB ester *P*	0.03	0.03	< 0.001	< 0.001	< 0.001

Table 5: Relative weight of small intestine (g/100 g live weight) and counts of major bacterial species (colony forming units, cfu/g digesta) in the small intestinal digesta at 42-d of age (means of 8 birds selected from each diet; one bird from a single pen)

[1]Control diet without any added growth promoter; [2] control diet supplemented with bacitracin methylene di salicylate 10%; [3] control diet supplemented with a glyceryl ester of butyric acid (Prophorce SR 130, Perstorp Holdings, Sweeden); [4] control diet supplemented with both AGP and TB ester. Means in a column bearing dissimilar superscripts vary significantly.

Supplementation of AGP and TB decreased counts of *Clostridium perfringens* in the T2, T3 and T4 groups as compared with that in the T1 group and AGP was superior to TB in this regard (main effect AGP $p < 0.001$, main effect TB $p < 0.001$, AGP x TB $p < 0.01$).

The data related to the mucosal architecture of the small intestine are presented in Table 6. Supplementation of the AGP had no effect on villus height ($P > 0.05$) and there was only numerical difference between the T1 and T2 groups in this regard. Supplementation of TB increased villus height in the T3 group ($P < 0.001$) substantially over that in the T1 and T2 groups. There was no interaction between AGP and TB (AGP x TB $P > 0.05$) and the incremental change in the villus height due to AGP and TB supplementation in the T4 group compared to the T3 group was only marginal. The effect of AGP and TB either alone or in combination on crypt depth, width of the villus and mucosal thickness was not significant ($P > 0.05$).

Antibody titer against ND and IBD at 12 and 30-d of age (Table 7) was not affected by dietary supplementation of AGP and TB either alone or in combination (main and interaction $p > 0.05$) indicating subtle effect of these treatments on post vaccination immune reactions.

Dietary groups	Villus height	Villus width	Crypt depth	Mucosal thickness
T1-Control[1]	1856.6[a]	280.9	37.2	375.2
T2-AGP[2]	2069.4[a]	253.8	27.1	385.3
T3-TB [3]	2983.2[b]	252.5	30.1	396.5
T4-AGP+TB [4]	3001.5[b]	266.2	34.7	421.0
Pooled SEM	111.05	10.98	2.34	16.53
Main effect AGP *P*	0.37	0.77	0.57	0.62
Main effect TB ester *P*	<0.00	0.73	0.96	0.41
AGP x TB ester *P*	0.45	0.38	0.13	0.83

Table 6: Histo-morphology of the small intestinal section (jejunum) at 42-d, µm (means of 8 birds selected from each diet; one bird from a single pen).

[1]Control diet without any added growth promoter; [2] control diet supplemented with bacitracin methylene di salicylate 10%; [3] control diet supplemented with a glyceryl ester of butyric acid (Prophorce SR 130, Perstorp Holdings, Sweeden); [4] control diet supplemented with both AGP and TB ester. Means in a column bearing dissimilar superscripts vary significantly.

Dietary groups	IBD titer		ND titer log2	
	12 d	30 d	12 d	30 d
T1-Control[1]	2300.9	5363.8	4.19	2.65
T2-AGP[2]	2307.1	5366.9	4.23	2.68
T3-TB [3]	2301.3	5376.5	4.15	2.70
T4-AGP+TB [4]	2302.0	5363.3	4.16	2.67
Pooled SEM	19.9	39.5	0.07	0.04
Main effect AGP *P*	0.93	0.95	0.87	0.97
Main effect TB ester *P*	0.96	0.96	0.74	0.80
AGP x TB ester *P*	0.95	0.92	0.93	0.75

Table 7: Antibody titer against Newcastle disease and infectious bursal disease at 12 and 30-d (means of 8 birds selected from each diet; one bird from a single pen).

[1]Control diet without any added growth promoter; [2] control diet supplemented with bacitracin methylene di salicylate 10%; [3] control diet supplemented with a glyceryl ester of butyric acid (Prophorce SR 130, Perstorp Holdings, Sweeden); [4] control diet supplemented with both AGP and TB ester.

Discussions

Dietary supplementation of SCFA especially that of TB, reportedly yielded beneficial effects in poultry which not only include antibacterial effects but also related to improved growth and development of gastrointestinal tract, providing energy source for the host post-absorption and stimulation of intestinal blood flow [20]. Previous studies have reported either positive [21,22] or positive, but not significant [13,23,24] effects of TB supplementation on poultry performance. In the present study BW and feed intake in the birds receiving AGP and TB supplementation did not differ significantly from that in the control group. Feed conversion, however, was numerically better especially in the TB and the TB +AGP groups, although this may not be enough to justify the inclusion of this additive in the feeding regime of broilers. As a matter of fact, the AGPs are included in the feed of poultry and other livestock to improve their productivity and health status. So, when the issue of replacing the AGPs comes into the fore the main question that haunts the producer pivots around maintaining the competitiveness of production without these additives. Butyric acid and its derivatives like the glycerides of butyrate are considered to be an effective alternative to the AGPs. Thus, the study was based on the hypothesis that supplementation of AGP or TB would improve the performance of the broilers compared with that in the control group and a further synergistic effect would be obtained when both were supplemented together. The results of the study did not support this hypothesis which is intriguing yet can be explained by the classical works of Muramatsu., *et al.* [25] who clearly showed that in germ free animals the antibiotic growth promoters had little role to play and antibiotics improve the growth of birds by sparing the energy which is otherwise consumed by the infectious organisms present in the gut and amounts to be about 10% of the total energy consumed. There are reports which indicate little effect of supplementation of different organic acids on performance of broilers s [26-29] and this effect has been attributed to the absence of any real enteric challenge [30].

There are enough bibliographic evidences that butyrate can improve body weight gain, voluntary feed intake and FCR of broiler chickens, although the literature shows large discrepancies in the effect of butyrate on growth performance (Moquet., *et al.* 2016). However, in the current study supplementation of TB had only subtle effect on BW and ADFI. It has been reported that health status of the animal, diet composition and environmental conditions influence the response of the broilers to butyrate supplementation (Cerisuelo., *et al.* 2014). This hypothesis is plausible since supplementation of BMD also yielded little effect on BW and ADFI which suggests that in absence of a real enteric challenge the gut acting growth promoting substances might have little role to play in augmenting broiler performance (Bedford, 2000). This challenge is made up of, among other things, the background micro flora present in the cage, pen or shed where the animal lives. Muramatsu., *et al.* [25] reported that germ-free birds offered the same diet grew substantially quicker and appeared to capture less energy from the diet than their conventional counterparts. The discrepancy in energy capture is a consequence of the micro flora extracting a significant amount of energy from the diet, energy which is not available to the bird. These data suggest that, in this experiment where semi-synthetic diets were fed, the 'energy cost' of the micro flora was at least 10% of the total apparent metabolizable energy. Antimicrobial growth promoters conserve this apparently lost energy and shunt that towards growth.

There might be other explanations for the lack of response to the dietary supplements especially to that of the butyrate. Antongiovani., *et al.* [23] reported that when mono-, di-, and try-glycerides of butyric acid were supplemented to broiler diets, body weight gain improved during the first week and the difference in body weight between the treatment and the control group was lost as the birds grew older. These workers concluded that butyric acid might work during the early phase of the life and not afterwards. Friedman., *et al.* (2005) highlighted that the development of adult-type GALT in the chicken occurs in early life, mostly in the first week, and the functional development of the intestine as a digestive and absorptive organ is closely related to the maturity of GALT. As such there are evidences that the functional maturation of enterocytes is driven by diet characteristics and that the development of functional GALT is consequent to the exposure to micro flora antigens. The antigenic stimulation comes from the enteric challenge and the environment where the birds are placed plays a very important role in this process since the intestine of the chicks is sterile before hatching (Bedford, 2000). The role of butyric acid is important here since following inoculation microbes degrade the structural carbohydrates of feed and produce SCFA of butyrate is the most important to promote the intestinal health of the bird (Friedman et al., 2005). The better FCR in the AGP and the AGP+TB groups on 14-d of age suggested positive effects of these supple-

ments during the initial phase of life and this is in agreement with the hypothesis mentioned above.

The statistical insignificance not-withstanding, FCR in both the AGP and the TB groups was numerically superior to that in the control group suggesting the enteric challenge, albeit sub-optimal to induce a paradigm shift in bird's response, brought about some responses in terms of FCR. In this study, weight of the small intestine was lower in the TB and the AGP+TB groups which is in agreement with the findings of Tonel., *et al.* [31] who reported that supplementation of butyrate in piglet diet significantly reduced weight of the small and large intestine. This particular observation was accompanied by a significant decline in the numbers of the potential pathogens (*E. coli*, *Salmonella* and *Clostridium perfringens*) in the groups receiving either AGP or TB or both which was in line with the findings of Abdelquader and Al-Fataftah [27] and Jahanian and Golshadi (2015) and suggestive of the potential that TB has on reducing the pathogen load. It should be noted here that the reports on the effects of TB on nutrient digestibility is rather positive. Smulikowska., *et al.* (2009) reported that dietary inclusion of fat coated butyrate at 0.3 g/kg significantly increased apparent total tract digestibility of nitrogen and organic matter without affecting crude fat digestibility in broiler chickens. In the same study, nitrogen retention was improved while apparent metabolizable energy content of the diet was unaffected by butyrate supplementation. Qaisrani (2014) reported a trend for higher proventricular proteolytic activity in broilers fed the butyrate derivative and this may be related to an improvement in protein digestibility. Jahanian and Golshadi (2015) reported an increase in ether extract digestibility due to supplementation of TB glycerides in laying hens. According to Moquet., *et al.* (2016) butyrate might improve protein digestibility whereas improvements in energy digestibility are uncertain. The improvement in digestibility might have a correlation with the positive effects of butyrate on histological structures of the small intestine. Leeson., *et al.* [13] reported that unlike antibiotics there is a possibility that butyrate helps in the maintenance of the intestinal villi structure. In the current experiment too supplementation of butyrate either alone or in combination with antibiotic significantly increased the height of the villi in the jejunum corroborating earlier studies [23,27]. However, it is difficult to interpret if the longer villi could facilitate nutrient digestibility since there were only numerical differences between the dietary groups with regard to BW and FCR. Although, long villi and short crypts are generally regarded as the indicator of healthy small intestine additional parameters like mucosal enzyme activity, mucus layer thickness and composition, or number and quality of goblet cells should also be considered while judging the digestive capacity of the small intestine. In the current study mucosal layer thickness, crypt depth and villus width were unaffected and hence it may not be inappropriate to assume that either of the supplementation had inadequate effect on nutrient digestion and thus had little effect on BW and FCR.

The data presented in Table 5 clearly suggests that antibiotics like BMD while controlling pathogens like *C. perfringens* also depopulate the good bacteria like the *Lactobacillus* while butyrate has not only the potential to control the potential gram negative pathogens and *C. perfringens* but also to favour the growth of *Lactobacillus*. The effect of butyrate on *Salmonella* is well documented [32,33] and there are reports which suggest that butyrate may reduce the relative abundance of *C. perfringens* in small intestine (Moquet et al., 2016) and the current findings are in line with these earlier reports. The mechanism by which butyrate exerted this effect is not very clear since the hypothesis which is valid for the products which get dissociated in the foregut itself will not be valid for the product used in the present experiment involving triglycerde of butyrate. Generally, if butyric acid gets dissociated in the proximal part of the gastro-intestinal tract, it may sustain development of a more beneficial microbiota in the distal segment of the small intestine [34]. Nevertheless, the butyrate used in this study might have had induced some changes in the digestion and absorption of nutrients and thus affected the microbial diversity of the distal segments of the small intestine [35].

The weight of the small intestine relative to the body weight is intriguing and suggests towards a significant effect of butyrate on this parameter. There are reports which suggest that butyrate down regulates the pro-inflammatory pathways inhibiting cytoplasmic IκB kinase activity [36]. The small intestine is always under controlled inflammation and down-regulation of pro-inflammatory processes spares nutrients for growth and skeletal development. However, even if the lower relative weight of the small intestine obtained in the present experiment is explained by the above mechanism it has to be accepted that this effect was not extrapolated to the other related parameters like the vaccine titers against ND and IBD and the performance traits.

It was concluded from the present study that glyceride of butyrate may be an effective tool to rear broiler chickens without using in-feed antibiotic growth promoters like BMD under standard management condition. Butyrate may increase the height of the villi in the small intestinal segments and reduce the load of gram-negative bacteria like *Salmonella* and *E. coli* as well as *C. perfringens* while increasing the counts of *Lactobacillus* in the small intestinal digesta. However, in absence of some real enteric challenges these effects might not be translated conspicuously into body weight and feed conversion ratio. The results of the study also revealed that there might be little benefit of supplementing butyrate along with an AGP although it is difficult to conclude on this finally unless studies involving a real necrotic enteritis challenge are performed.

Authors' Contributions

Conceived and designed the experiments SH, DV, AS. Performed the experiments and contributed materials/analytical tools: AS, SD, IS, AD. Analysed the data: SH. Wrote the paper: SH, DV.

Competing Interests

The authors declare that no competing interest exists.

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