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RESEARCH ARTICLE

In Vitro and *In Vivo* Anticoccidial Effects of Butyric Acid and Its Impact on Blood and Serum Chemistry of Broiler Chickens

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Abstract

Present study was aimed to evaluate the anticoccidial activity of butyric acid by both in vitro and *in vivo* methods. *In vitro* trial was conducted by sporulation inhibition assay to examine the influence of butyric acid on sporulation and damage of coccidian oocysts. Administration of various concentrations of butyric acid induced sporulation inhibition and also damaged coccidian oocysts in dose dependent manner. For *in vivo* trials, 72 (day-old) broiler chicks were randomly divided into 6 groups i.e., A, B, C, D, E and F having equal chicks in each group (n=12). After one week of acclimatization, three doses of butyric acid 1.2%, 1% and 0.8% were given to group A, B and C, respectively while group D was named as positive control (infected medicated), group E was named as negative control (infected and non-medicated) and group F served as normal control (non-infected) group. On the same day, all treated groups were orally infected with 50.000 sporulated oocysts of *Eimeria tenella*. Results revealed that administration of butyric acid induced positive effect on chicken's performance such as weight gain, FCR and anticoccidial parameters like lesion and oocysts score, oocyst per gram. Butyric acid also improved hematological values and serum chemistry of broiler chicken.

Keywords: *Butyric acid, Eimeria, Treatment, Anticoccidial effect, Broiler, Chicken*

Butirik Asitin Etlik Piliçlerde *In Vitro* ve *In Vivo* Antikoksidiyal Etkinlikleri ve Kan ve Serum Kimyası Üzerine Etkisi

Öz

Bu çalışmada, bütirik asitin antikoksidiyal aktivitesinin in vitro ve in vivo yöntemlerle değerlendirilmesi amaçlandı. Bütirik asitin, koksidian ookistlerin sporülasyonu ve hasarı üzerine etkisini incelemek için in vitro sporülasyon inhibisyon testi yapıldı. Çeşitli konsantrasyonlarda bütirik asit uygulanması sporülasyon inhibisyonuna neden olurken, doza bağlı bir şekilde koksidian ookistlere zarar verdi. In vivo denemeler için 72 adet bir günlük etlik civciv, her grupta eşit (n=12) civciv olacak şekilde rastgele 6 gruba, yani A, B, C, D, E ve F'ye ayrıldı. Bir haftalık uyum aşamasından sonra grup A, B ve C'ye sırasıyla %1.2, %1 ve %0.8 bütirik asit verilir iken, grup D pozitif kontrol (enfekte ve ilaçlı), grup E negatif kontrol (enfekte ve ilaçsız) ve grup F normal kontrol (enfekte olmayan) grubu olarak değerlendirildi. Aynı gün, tedavi edilen tüm gruplar, *Eimeria tenella*'ya ait 50.000 sporlu ookist ile oral yolla enfekte edildi. Sonuçlar, bütirik asit uygulamasının piliçlerin kilo alımı ve FCR gibi performans ve lezyon ve ookist skoru ve gram başına ookist miktarı gibi antikoksidiyal parametreler üzerine olumlu etkilere yol açtığını ortaya koydu. Bütirik asit ayrıca broyler piliçlerin hematolojik değerlerini ve serum kimyasını da iyileştirdi.

Anahtar sözcükler: *Butirik asit, Eimeria, Sağaltım, Antikoksidiyal etki, Broiler, Tavuk*

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Introduction

In Pakistan, poultry sector contributes to enhance the economy of country by generating employment for about 1.5 million people as well as in meat production sectors contributing 19% of the total meat [1-3]. However, many factors especially diseases play an important role in limiting the poultry production [4,5]. Commercially, poultry farms dealing with broiler chickens are facing many parasitic disorders including coccidiosis, caused by species of *Eimeria* which are obligate protozoans that invade intestinal lining of chicken [6,7], causing severe gastrointestinal damages leading to high mortality rates [8,9]. Moreover, these infected sporozoites induce severe damages like loss of body weight, hemorrhage feces, reduced efficacy and ultimately death of the chicken resulted in serious worldwide economic losses approximately more than 3 billion US dollars [10-12].

Since a long time, many unique anticoccidial drugs are being used in order to treat coccidiosis and their persistent use has caused the drug resistance factor in the birds [13,14]. Due to anticoccidial drug resistance, researchers are trying to find alternative strategies to control coccidiosis in chickens [15-17]. Among alternatives, different acids have shown to improve intestinal health and excellent anti-coccidial effects. Short-chain fatty acids such as butyrate are considered as potential alternatives to antibiotic growth promoters [18,19]. Butyric acid (butyrate) has potential to improve intestinal health [20] and growth stimulant along with bactericidal agent because its activity increases in undissociated form [21,22]. Hence, current study was designed to evaluate the *in vitro* and *in vivo* anticoccidial effects of butyric acid (butyrate) in broiler chickens.

Material and Methods

Acid Collection and Verification

Butyric acid was obtained from an authentic chemical company (Sigma-Aldrich, Pakistan) and was further verified and compared with already stored samples in the Department of Chemistry, University of Agriculture Faisalabad, Pakistan.

Parasite

Eimeria tenella oocysts were collected from the cecal portion of chicken guts and kept in 2.5% sodium hypo-chlorite in the ratio of 4:1 for the time interval of 25 min. Standard method of sedimentation was used for isolation of *E. tenella* oocysts. During sedimentation, desiccators were used and filled with water for four times to get the final results for the evacuation of the coccidial oocysts. Further sporulation of isolated oocysts was done in 2.5% potassiumdichromate solution at 25-29°C and by maintaining 60-80% humidity [23]. The sporulation of the oocyst was confirmed by examining sporocysts under light microscope at 40X.

In Vitro Trial

In vitro experimentation was conducted by sporulation inhibition assay (SIA) to examine the influence of butyric acid against the sporulation of coccidian oocysts. For this purpose, unsporulated oocysts were maintained in petri dishes filled with 2.5% solution of potassium dichromate followed by the thickness of 6 mm and exposed to six different concentrations of butyric acid (10, 5, 2.5, 1.25, 0.625 and 0.31% w/v) in three replicates. Potassium dichromate solution and dimethyl sulfoxide (DMSO) were kept as the control groups. Unsporulated oocysts were incubated at 25-29°Cwith butyric acid for 48h. After incubation, tap water was used to wash and then kept at 4°C before counting. The percentage of sporulation was calculated by counting sporulated and non-sporulated oocysts out of 40 oocysts. The effect of butyric acid on morphology of *E. tenella* oocysts was also examined in terms of size and shape. The cover slip was pressed slightly to keep the oocysts pressured for better illustration of their morphology under the microscope.

In VivoTrial

Seventy-two days old broiler chicks (Hubbard Al-Noor Chicks, Pvt) were purchased from local hatchery and raised under controlled management practices with division into 6 groups having A-C treated with butyric acid 1.2% or 1% and 0.8% through diet (12 chicks in each group) and fed with coccidian free diet, Group D (positive control) was maintained as infective and treated control; Group E (negative control) was maintained as infective and non-treated control while Group F (control) was maintained as non-infective and non-treated control. At the same day, all treated groups were orally infected with 50.000 sporulated oocysts of *E. tenella*. Chicks were subjected to vaccination against New Castle Disease (ND) at 5th day while the vaccination for Infectious Bursal Disease (IBD) was administrated on 8th and 16thday. During one week of acclimatization, chicks were kept at 27-32°C with reduction in each week along with standard light conditions for 24 h.

Anticoccidial Parameters

Anticoccidial activity of butyric acid was assessed by following parameters including:

- Weight Gain and Feed Conversion Ratio

Weekly weight change was assessed from each group until the completion of the experiment. Feed Conversion Ratio (FCR) of birds was calculated by following formula.

FCR = Mean Feed Consumption/Mean Weight (g)

- Lesion and Oocyst Score

On 7th day post inoculation lesion scoring technique was used for that score on a scale of 0 to 4 [23]. At 7th day of

infection, oocysts were scored on a scale of 0 to 5 was done by following the already described method [24].

- Oocyst Per Gram of Feces (OPG)

McMaster technique was used to analyze oocyst per gram of fecesby mixing 3 g of feces with 42 mL of saturated NaCl in beaker (Pyrex) [25]. The oocyst per gram feces (OPG) was calculated by the formula given below.

OPG = oocyst count - dilution factor (fecal sample volume/ counting chamber volume)

Hematological Analysis

Packed cell volume determinations (PCV), red and white blood cell count and haemoglobin (Hb%) level were assessed by using hematology analyzer FMI-6180 (Jiangsu, China) by following the standard method as reported previously [26].

Serum Analysis

Commercially available kits (Fortress Diagnostic Ltd. UK) were used to analyze the serum biochemical parameters such as Alanine transferase (ALT), Aspirate aminotransferase (AST), Urea, Creatinine, Lactate dehydrogenase (LDH) by following standard method provided by the supplier.

Statistical Analysis

ANOVA (analysis of variance) was conducted on different parameters. Furthermore, Duncan's multiple range test was performed to evaluate the significance for the groups. P<0.05 was reported as statistically significant.

Results

The statistical analysis of all treated groups presented that administration of various concentrations of butyric acid significantly affected the sporulation process of *Eimeria* oocysts as compared to control groups (P<0.05) (*Table 1*).

Butyric acid was also effective in damaging internal and external morphology of *Eimeria* oocysts in concentration-dependent manner as compared to both control groups (P<0.05) as indicated in *Table 2*.

Effect of butyric acid on body weight gain of all treated groups was significantly different to infected non medicated group (P<0.05). Administration of butyric acid also improved FCR of all the treated groups. Statistical analysis of FCR was not conducted due to group feeding of birds, presented in *Table 3*. Administration of various doses of butyric acid significantly reduced oocysts per gram of feces (OPG)and lesion scores which were significantly different to infected non medicated group (P<0.05). The results are shown in *Table 4*.

Table 1. *Efficacy of various concentrations of butyric acid on sporulation of oocysts (%)*

Groups	Treatments (%)	Sporulation (%)
Control-I	0.00	75.00±2.517^{b}
Control-II	0.00	80.00±2.082^{a}
BA (10%)	10.0	20.00±1.155^{f}
BA (5.00%)	5.00	30.00±1.000^{e}
BA (2.50%)	2.50	47.90±1.528^{d}
BA (1.25%)	1.25	48.00±1.000^{d}
BA (0.62%)	0.62	55.00±1.732^{c}
BA (0.31%)	0.31	58.65.00±0.665^{c}

BA: Butyric Acid. Means that bear different letters are statistically significant (P<0.05)

Table 2. *Efficacy of various concentrations of butyric acid on damage of oocysts (%)*

Groups	Treatments (%)	Damage(%)
Control-I	0.00	0.00±0.00^{e}
Control-II	0.00	0.00±0.00^{e}
BA (10%)	10.0	40.30±0.88^{d}
BA (5.00%)	5.00	50.32±1.20^{c}
BA (2.50%)	2.50	58.60±1.45^{b}
BA (1.25%)	1.25	60.00±0.57^{b}
BA (0.62%)	0.62	65.65±0.88^{a}
BA (0.31%)	0.31	68.00±1.15^{a}

BA: Butyric Acid. Means that bear different letters are statistically significant (P<0.05)

Table 3. *Efficacy of various concentrations of butyrate on weight gain and FCR*

Groups	Body Weight (g)	FCR (g/g)
A	355±3.21^{c}	2.6
B	377±4.35^{b}	2.5
C	387±4.35^{ab}	2.1
D	402 ±5.50^{a}	2.4
E	292±4.66^{d}	2.8
F	385±5.19^{ab}	2.2

Means that bear different letters are statistically significant (P<0.05)

Table 4. *Effect of various concentrations of butyric acid on OPG and Lesion score*

Groups	OPG *	OPG **	Lesion Score
A	77.60±1.9^{b}	50.82±3.14^{b}	3.00±0.57^{ab}
B	70.39±1.5^{bc}	45.86±0.87^{bc}	2.33±0.33^{bc}
C	65.71±1.4^{c}	39.40±1.05^{c}	1.33±0.33^{cd}
D	64.21±2.2^{c}	39.48±1.38^{c}	1.00±0.00^{de}
E	95.69±3.4^{a}	75.73±2.51^{a}	4.00±0.57 ^{a}
F	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{e}

A: *1.2%;* ***B:*** *1.00%;* ***C:*** *0.8%;* ***D:*** *Infected medicated;* ***E:*** *Infected non-medicated;* ***F:*** *Normal;* ***OPG*:*** *Oocysts per gram of feces 7th day post infection;* ***OPG**:*** *Oocysts per gram of feces 14th day post infection*

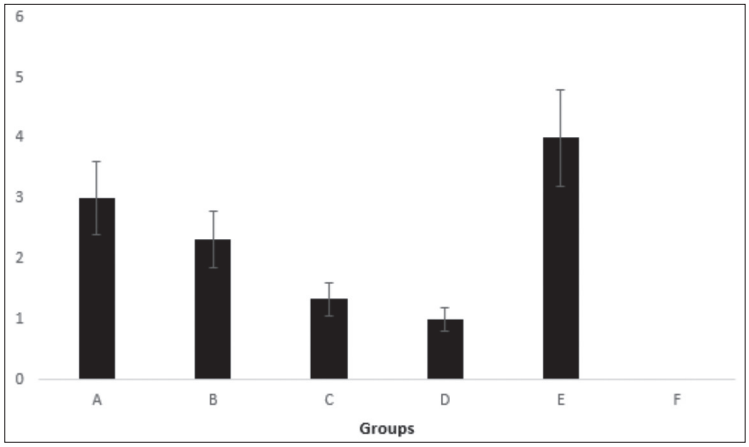
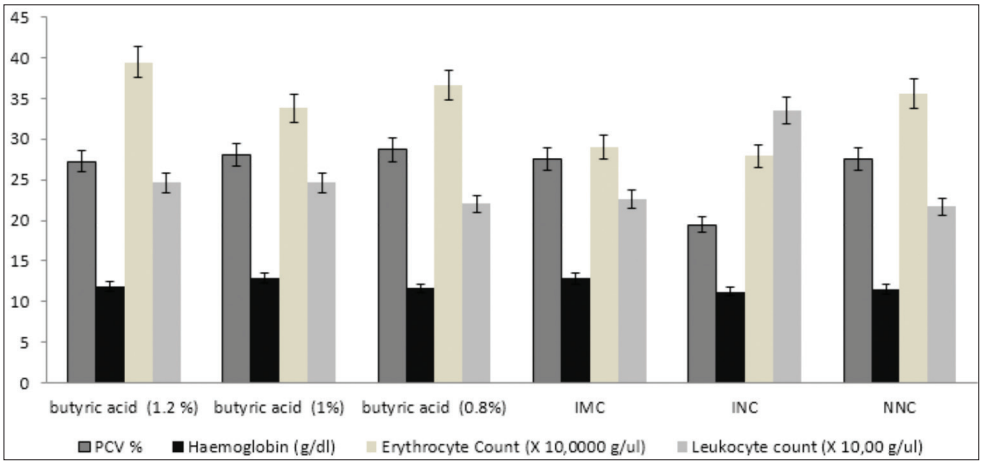


Fig 1. Effect of various concentrations of butyric acid on oocysts score of chickens mixed with *Eimeria tenella*

A: 1.2%; **B:** 1.00%; **C:** 0.8% **D:** Infected Medicated; **E:** Infected non-Medicated; **F:** Normal

Fig 2. Effect of various concentrations of butyric acid (butyrate) on various blood parameters of broiler chicken



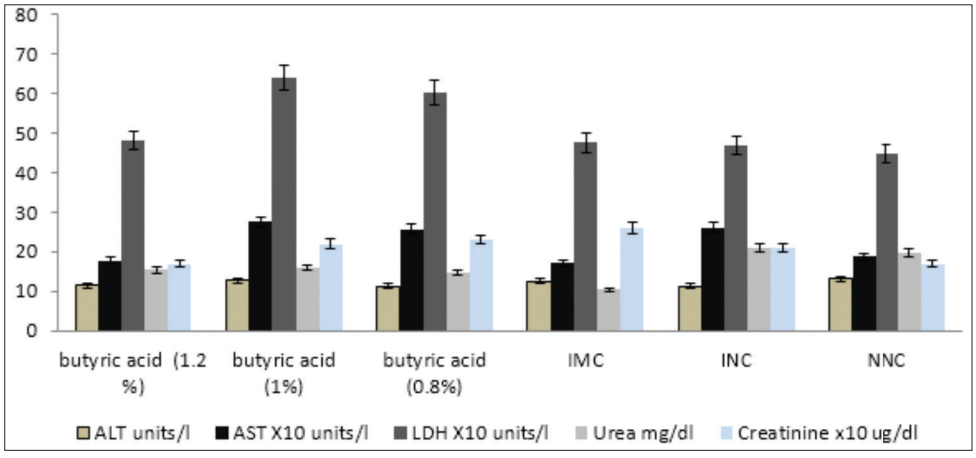


Fig 3. Effect of various concentrations of butyric acid (butyrate) on various serum parameters of broiler chicken

Oocyst score in butyric acid treated groups was also significantly different to infected non medicated group ($P<0.05$) as indicated in *Fig. 1*. Current research studied the effect of various doses of butyric acid on the hematological parameters i.e., Hb, PCV, WBCs and RBCs of broiler chicken mixed with *E. tenella*. Butyric acid treated groups improved hematological parameters which were significantly different to infected non medicated group ($P<0.05$), the results are presented in *Fig. 2*. The effect of various doses of butyric acid on serum enzyme like ALT, AST, urea, creatinine and LDH were significantly different ($P<0.05$) from other groups *(Fig. 3)*.

Discussion

Many recent and previous studies have demonstrated remarkable anticoccidial and growth promoting effects of various organic acids and other novel compounds [27-29] against avian coccidiosis [2,7,8,13,14,30]. Current study has demonstrated *in vitro* and *in vivo* anticoccidial effect of butyric acid. Butyric acid showed inhibitory effect on sporulation process and also damaged *Eimeria* oocyst. Similar types of findings have beenreported in a recent study in which *Vitis vinifera* extract showed *in vitro* anticoccidial

activity in dose dependent manner [6]. The administration of various doses of butyric acid in diet improved weight gain and feed conversion ratio of infected chickens. Positive effect of different organic acids like butyric acid on weight gain and feed conversion of infected birds could be due to their complementary synergistic effect resulting from supplementing diets with a mixture of these additives, especially under the stressful conditions imposed by *Eimeria*. The reported results are in line with previous studies [16-18].

Moreover, in the present research, treatment with various doses of butyric acid induced protective effect by lowering oocysts per gram of feces (OPG), lesion and oocyst score in broiler chicken. Likewise, the different concentrations of acetic acid reduced negative performance and pathogenic effects (lesion and oocyst score) associated with *E. tenella* challenge in broiler chickens [28]. In another study, similar type of anticoccidial effect of hydrochloric acid (HCl) has been reported. Administration of hydrochloric acid in water reduced lesion and oocyst score against *E. tenella* infection in broiler chickens and also improved weight gain and feed conversion ratio of birds [29].

Different organic acids have been reported for their positive effect on performance parameters and have also shown therapeutic effects against coccidiosis. Previously, dietary supplementation of 0.2% butyric acid at the concentration of 0.2% improved the growth performance and carcass quality of broiler chicks experimentally challenged with coccidiosis [30]. Different studies are warranted on the effects of butyrate on epithelial cell development in the small intestine of young broilers and suggested butyrate the best candidate for improving performance of broiler chickens [31]. Likewise, antimicrobial and antibacterial activities of various acids as additives to enhance the protective effect of anticoccidial drugs have also been reported in broiler chickens [32,33].

Findings of current studies are similar with the research outcomes of previous studies [34,35] who found similar effect of butyric acid on serum chemistry of broiler chickens. Moreover, present findings of butyric acid on serum enzymes levels (ALT and AST) were also observed in previous studies [34,35]. Hematological parameters were also improved in infected birds as reported previously [36] where similar trend of butyrate effect on red and white blood cells count was observed in broiler chicken.

In a recent study, maslinic acid found in plant *Olea europaea* showed the remarkable increase in weight gain and reduced the lesion score in chickens infected with *E. tenella*. Histopathological studies showed a significant decrease in the infection rate in the chicks treated with maslinic acid [37].

In one study, the three products of organic acids including Acidomix® (ammonium formate, formic acid, ammonium propionate), Activate® (calcium butyrate, fumaric acid, benzoic acid) and Lacplus® (lactic acid, citric acid, fumaric acid) induced protective immunity, improved intestinal health and reduced lesion and oocyst score against *E. tenella* infected broiler chickens [38].

Most recently, butyric acid (tributyrin) has been reported to improve weight gain and immunity against *Eimeria maxima* infected broiler chicken [39]. Additionally, dietary supplementation of formic acid (0.5%) and propionic acid (0.5%) produced positive effects in terms of growth performance, gut microbiota and pH, carcass traits, and immune response in broiler chickens. Dietary supplementation with formic or propionic acids positively influenced the production parameters, but a mixture of both acids produced more effects in broiler chickens [40].

Present research concluded that treatment with different doses of butyric acid induced protective effect on body weight, lesion score, FCR value and oocysts per gram of feces. Moreover, induction of butyric acid also induced dose dependent protective effect on hematological parameters (RBCs, WBCs, Hb, PCV) and serum chemistry (ALT, AST, Urea, Creatinine, LDH) in the *E. tenella* infected chickens. However, further research is needed for the development of acid based anticoccidial product especially from butyric acid.

Author Contributions

ZR, RZA, AA, ZS planned and designed the research. TR, RH, KM helped in execution of the research trials, data collection and analysis. AR and KH assisted in hematology and serum chemistry.

Conflict of Interest

The authors declared that there is no conflict of interest between the authors.

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