OPEN ACCESS
Advances in Animal and Veterinary Sciences

Research Article

Effectiveness of Phyto-biotic Fortified-Diet on Broilers Growth Performance and Gut, Cellular, and Humoral Immunity Signals

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Abstract | The current research was designed to examine the effectiveness of Phyto-biotic on broilers performance and immunity as well as liver and kidney functions. Ninety, one-day-old Cobb chicks were allocated into two equal groups (n=45) with three replicates per group. The first group, the control (G_1) fed basal diet only while the second one (G_2) fed the basal diet supplemented with 0.5gm/kg diet Phyto-biotic. Experimental birds were monitored for 6 weeks old. The growth performance parameters were determined. The serum levels of aspartate and alanine aminotransferases (AST and ALT), creatinine, and uric acid were estimated at 21thand 42nddays of age. The cellular immunity was examined at the third and fifth weeks. Genes expression of the gut immunity (IL4 and IFN-γ) were assessed in cecal tonsils and spleen at 35th days of the study. Humoral antibody titers against Newcastle Disease (ND), Avian Influenza (H5N1), and Infectious Bronchitis (IB) viruses vaccines were evaluated at 28th and 42nd days of the study. The dietary inclusion of Phyto-biotic in broilers resulted in significant improvement of their growth performance, liver and kidney functions, phagocytic index and percent, and serum antibody titers against ND virus. Together with, a significant up-regulation of the genes expression of IL4 and IFN-γ in the cecal tonsils and spleen. In conclusion, the Phyto-biotic could be effectively used as a growth promoter and immune-stimulant in broilers breeding for their role in the improvement of growth performance and immunity with no deleterious effect on the liver and kidney functions.

Keywords | Growth promoters, Broiler, Essential oil, Gut cytokines, Immuno-stimulants, Phyto-biotic.

Editor | Kuldeep Dhama, Indian Veterinary Research Institute, Uttar Pradesh, India.
Received | October 29, 2018; **Accepted** | November 23, 2018; **Published** | April 10, 2019
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Citation | El-Sheikh SM, Abdalim AF, Moursi MK, Helal MS, Ahmed E (2019). Effectiveness of phyto-biotic fortified-diet on broilers growth performance and gut, cellular, and humoral immunity signals. Adv. Anim. Vet. Sci. 7(6): 466-473.
DOI | http://dx.doi.org/10.17582/journal.aavs/2019/7.6.466.473
ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331

INTRODUCTION

The broiler production had been growing rapidly in the last few decades for the increased demand to provide a safe and cheap source of animal proteins (Yatao et al., 2018). This situation directed the broiler producers to the intensive rearing. Unfortunately, this intensive system led to the development of several challenges as overcrowding, rapid diseases spreading, lower immunity, and further economic losses (Salim et al., 2018). To overcome these problems, the producers were trended toward using antibiotics for either treatment or prevention of the infectious diseases and as an easy way for growth promotion (Costa et al., 2017). However, large number of research clarified that excessive inclusion of antibiotics in animals feed will intensify the potential risk of bacterial resistance in human. Therefore, this bacterial resistance and antibiotic residues in animal products led to raising the concern in using antibiotics as growth promoters and finally resulted in the ban on using in-feed antibiotics worldwide. The banning of antibiotics use in the poultry production encourages the researchers to search for substitutes which have the same effect of antibiotics or more potent without the adverse end results of antibiotics. These alternatives should be natural, non-toxic, and cause no residue in poultry meat such as probiotics, prebiotics, synbiotics, acidifiers, and Phy-

to-biotics (Al-Yasiry et al., 2018).

Phyto-biotics can be defined as "plant-derived compounds incorporated into diets to improve the productivity of livestock through redeeming the feed properties and also to enhance the quality of food derived from those animals" (Zheng et al., 2009). Thus, they are feed additives that could have good effects on either growth performance, immunity or production of superior quality products which have a great interest nowadays as it is an organic product (Mohammadi et al., 2018). The dietary incorporation of essential oils in broiler feeds resulted in proper growth performance and production via improvement of feed intake probably because of improved palatability of the diet, also improvement of food quality through their antioxidant efficacy and antimicrobial action that led to a final reduction of intestinal pathogen pressure, enhancement of the digestibility of nutrients and improvement of gut tissue morphology (Hernandez et al., 2004). In addition to, Phyto-biotics ability to improve the host immunity via regulation of the gut-associated lymphatic system (Frankič et al., 2009). In this context, the current study was designed to clarify the strength of Phyto-biotic bioactive components on broiler growth performance and, liver and kidney functions as well as cellular and humoral immunity.

MATERIALS AND METHODS

Birds Management and Diets

Ninety, one-day old Cobb broiler chicks were obtained from El-Watania Poultry Company, Wadi El- natron, Egypt. The birds were housed on the deep-litter floor system. The temperature was set at 32°C for the first week of age and then decreased by 2°C per week till reach 22°C at 6th week of age. Relative humidity was established at 50-60% during the study. Feed and water were provided *ad libitum*. Diet was formulated as starter and grower-finisher to meet the nutritional requirements as recommended by the National Research Council (NRC, 1994) as shown in Table (1). The chicks were vaccinated according to the vaccination program illustrated in Table (2). This study was carried out in strict accordance with the guide for care and use of animals and the protocol was approved by the Ethical Committee of Animal Experiments at Zagazig University.

Feed Additive

The Phyto-biotic used was Digestarom PEP® (Biomin GmbH, Austria). This Phyto-biotic containing a mixture of essential oils including oregano oil (carvacrol and thymol), anise oil, and citrus oil.

Experimental Design

The chicks were allocated randomly into two groups (45, each). Each group was sub-divided into three replicates (n=15). The first group was the control (G_1) that received basal diet and second was the Phyto-biotic-treated group (G_2) that fed on basal diet plus Phyto-biotic at a concentration of 0.5g/kg feed (Diaz-Sanchez et al., 2015). This experiment was conducted for 6 weeks.

Growth performance parameters: Final body weight, total weight gain, total feed intake, total food conversion ratio (FCR), and total feed efficiency were estimated at the end of the experiment according to Awad et al. (2009b).

Table 1: The formulation of the basal diet for broilers

Ingredients kg/100kg	Starter (0-3 Weeks)	Grower-finisher (3-6 Weeks)
Yellow corn	58.57	64
Soyabean meal (48%)	32	25
Corn gluten (60%)	5	5
Soybean oil	0.7	2.5
Di-calcium phosphate (22%Ca&19%Ph)	1.5	0.95
Lime stone (35% ca)	1.7	1.74
Common salts	0.2	0.15
Methionine (95%)	0.13	0.36
Lysine (98%)	0	0.1
Vitamins and mineral premix**	0.2	0.2
Calculated compostion		
Protein (%)	23	20
k. calory ME/kg	2950	3120
Calcium (%)	1	0.9
Phosphorus (%)	0.48	0.35

** Each 2 kg contains the following vitamins and minerals: Vit. A 12 mIU, vit. D_3 2 mIU, vit. E 1000mg, vit. k_3 1000mg, vit. B_1 1000mg, vit. B_2 5000mg, vit. B_6 1500mg, vit. B_{12} 10mg, biotin 50mg, pantothinic acid 10g, nicotinic acid 30g, folic acid 1000mg, manganese 60g, zinc 50g, iron 30g, copper 4g, iodine 300mg, selenium 100mg, cobalt 100mg, carrier($CaCO_3$) to 3kg. (premix- Agrivet Pharm Elasher, Egypt. The diet was formulated in EL-Yomen Co., at Ismailia-Portsaid road.

Sampling

Blood sampling: At 21st 28th, 35th, and 42nd days, blood samples were collected from birds into heparinized and un-heparinized tubes. Six birds per group (two birds per-replicate) were sacrificed. The un-heparinized blood samples were centrifuged at 4000 rpm for 15 minutes to obtain serum. The collected serum were kept at -20 °C until used for biochemical parameters analysis. The heparinized blood samples were used for immunological assesement.

Tissue Sampling: Cecal tonsils and spleen were rapidly dissected out and rinsed with NaCl 0.9% at fifth week of

Table 2: The vaccination program

Age/day	Vaccine	Trade name	Company	Administration
6th day	ND& IB	Hitchner IB	Izo S.p.A, Italy	Eye drop
7th day	ND& IB	Inactivated ND&IB vaccine	(Intervet, Holland)	Sub/cutaneous injection
8th day	AI (H5N1)	Inactivated AI(H5N1) vaccine	(Marial, Spain)	Eye drop
14th day	Gumboro	Bursine® IBD	(Pfizer, USA)	Eye drop
20th day	ND& IB	Nobilis® clone 30 &MA5	(Intervet, Holland)	Drinking water
24th day	Gumboro	Bursine® IBD	(Pfizer, USA)	Drinking water

ND= Newcastle Disease, IB=Infectius Bronchitis, and AI(H5N1)= Avian Infleunza.

age.

The tissues were snap frozen then stored at -80 until used for genes expression analysis.

Liver and Kidney Functions

The collected serum at 21th and 42nd days were biochemically examined for quantitative kinetic estimation of the serum levels of AST and ALT according to the method described by Klauke et al. (1993). Additionally, creatinine and uric acids were determined according to the methods of Donsbough et al. (2010).

Immunological Analysis

Cell-mediated immunity: Cell-mediated immunity was examined by measuring the phagocytic index and percent of the blood macrophages using *Candida albicans* yeast *in vitro* at the age of 21st and 35th days of the experiment (Chu and Dietert, 1989).

Humoral immunity: The humoral immunity was determined at 28th and 35th days by determining the serum antibody titer against ND virus and AI (H5N1) using the hemagglutination inhibition test (Alexander and Chettle, 1977). While the serum antibody titers against IB virus was estimated by using commercial licensed ELISA kits prepared in National Laboratory for Veterinary Quality Control on Poultry Production (NLQP), Dokki, Giza, Egypt through a technique described by Morrow (2008).

Gut and spleen immunity: Gut immunity was evaluated by quantitative measurement of gene expression of IL4 and IFN-γ in the cecal tonsils and spleen at the age of 35th days. The intestine was taken carefully and the cecal tonsils and spleen were dissected and kept in -80°C until analysis after slaughter. The RNA extraction, preparation, and cycling conditions for real-time PCR were performed as described by Sławinska et al. (2014). Primers and probes used in real-time PCR were demonstrated in Table (3) (Markowski-Grimsrud and Schat 2003; Suzuki et al., 2009). Amplification curves and Cycle Threshold (CT) values were assessed by the stratagene MX3005P software. To estimate the variation of gene expression on the RNA level of the distinct samples, the CT of each sample was compared with that of the control group according to the "$^{\Delta\Delta}$Ct" method stated by Yuan Yuan et al. (2006).

Statistical Analysis

Data were tested for variance homogeneity distribution by SPSS. The data were statistically analyzed by analysis of variance method (ANOVA) using SPSS 18.0 Inc. 2009 software. Duncan multiple range test were used to compare the means. Data were presented as means ± standard error of mean and a probability value was considered statistically significant at (P< 0.05).

Table 3: Primer sequences for genes expression analysis

Gene	Primer sequences (5'-3')	References
IL-4	AACATGCGTCAGCTCCTGAAT. TCTGCTAGGAACTTCTCCATTGAA. (FAM) AGCAGCACCTCCCTCAAGGCACC (TAMRA).	
IFN-γ	AAACAACCTTCCTGATGGCGT. CTGGATTCTCAAGTCGTTCATCG. (FAM) TGAAAGATATCATGGACCTGGCCAAGCTC (TAMRA).	(Markowski-Grimsrud and Schat, 2003)
28SrRNA	GGCGAAGCCAGAGGAAACT. GACGACCGATTTGCACGTC. (FAM) AGGACCGCTACGGACCTCCACCA (TAMRA).	(Markowski-Grimsrud and Schat 2003; Suzuki et al. 2009)

RESULTS

Effect of Phyto-Biotic on Growth Performance

The Phyto-biotic fed group showed a significant increase (P<0.05) in the final body weight (2786.71*vs* 2150.17g), total weight gain, and total feed efficiency, together with,

a significant decrease (P<0.05) in the total feed conversion ratio (1.7 *vs* 2.16) compared to the group fed the basal diet only (Table 4). However, there was no significant difference in the total feed intake between groups.

Table 4: Effect of Phyto-bioticon the body performance of broilers

Parameters	Control	Phyto-biotic
Final body weight (gm)	2150.17±34.6[b]	2786.71±17.6[a]
Total body weight gain (gm)	2104.7±33.8[b]	2740.49±16.1[a]
Total feed intake (gm)	4636.7±38.4[a]	4730±75.1[a]
Total feed conversion ration	2.16 ±0.03[a]	1.7±0.04[b]
Total feed efficincy	0.46±0.006[b]	0.59±0.01[a]

All values are expressed as means ± standard error (SE); n=6. Means at the same row with dissimilar superscripts are statiatically different (P<0.05).

Effect of Phyto-biotic on Liver and Kidney Functions

Table (5) showed that the broilers fed on the diet supplemented with Phyto-biotic displayed a significant decrease (P<0.05) in the serum ALT level than those received the basal diet at both 21 and 42 days of age. Concerning the kidney function, the Phyto-biotic treated group possessed a significant decrease (P<0.05) in serum uric acid level than the control group at 21 days of the study.

Table 5: Effect of Phyto-bioticon the liver and kidney functions of broiler at 21 and 42 days

Parameters	Time	Control	Phyto-biotic
ALT (u/l)	21 days	5.03±0.15[b]	3.65±0.26[c]
	42 days	5.32±0.34[b]	3.78±0.21[c]
AST (u/l)	21 days	201.33±5.3[b]	204 ±4.64[b]
	42 days	204.67±5.58[b]	202.5±4.4[b]
Creatinine (mg/dl)	21 days	0.51±0.05[a]	0.58±0.03[a]
	42 days	0.46±0.03[a]	0.47±0.06[a]
Uric acid (mg/dl)	21 days	5.75±0.28[b]	4.15±0.24[c]
	42 days	6.4±0.28[b]	5.98±0.51[b]

All values are expressed as means ± standard error (SE); n=6. Means at the same row with dissimilar superscripts are statiatically different (P<0.05).
ALT= Alanine aminotransferase; AST= Aspartate aminotransferase.

Effect of Phyto-biotic on Cell-mediated Immunity

The dietary supplementation with Phyto-biotic caused a significant elevation (P<0.05) in the phagocytic index (5±0.14 *vs*3.28)and percent (77.83±2 *vs* 60.5±1.5) at 21 days than the un-supplemented group (Table 6). While there were no significant differences between groups in the phagocytic index and percent at the 35days.

Effect of Phyto-biotic on Humoral Immunity

Phyto-biotic fortified group showed a significant increase (P<0.05) in the antibody titer against ND at 42 days and H5N1 virus at 28 days of the study, respectively than the control group (Table 7). Nevertheless, there was no significant difference between groups in the antibody titer against IB virus.

Table 6: Effect of Phyto-bioticon phagocytic index and phagocytic percent of broiler at 21 and 35 days

Parameters	Time	Control	Phyto-biotic
Phagocytic index	21 days	3.28±0.11[b]	5±0.14[a]
	35 days	3.17±0.17[a]	3.25±0.1[a]
Phagocytic percent	21 days	60.5±1.5[b]	77.83±2[a]
	35 days	60.17±0.95[a]	62.67±2.04[a]

Values are expressed as means ± standard error (SE); n=6. Means within the same row with different superscripts are significantly different (P<0.05).

Table 7: Effect of Phyto-biotic on antibody titers against ND, H5N1 and IB of broiler at 28 and 42 days of the study

Parameters	Time	Control	Phyto-biotic
ND	28 days	3.5±0.43[a]	4.33±0.49[a]
	42 days	4.67±0.62[b]	7.67±0.42[a]
H5N1	28 days	3.5±0.43[b]	5.17±0.47[a]
	42 days	4.17±0.48[a]	3.83±0.79[a]
IB	28 days	3564.2±49.3[a]	3736.7±47.4[a]
	42 days	9258±182[a]	9472±171[a]

Values are expressed as means ± standard error (SE); n=6. Means within the same row with different superscripts are significantly different (P<0.05).
ND= Newcastle Disease; H5N1= Avian Influenza (H5N1); IB= Infectius Bronchitis.

Effect of Phyto-biotic on Gut and Spleen Immunity

Dietary inclusion of the Phyto-biotic caused a significant up-regulation (P<0.05) of the gene expressions of IL4 and IFN-γ in the cecal tonsils and spleen at 35 days of the study than the basal diet fed group as shown in Table 8.

Table 8: Effect of Phyto-biotic on the gene expression of IL4 and IFN-γ in both cecal tonsils and spleen at the age of 35 days

Organ	Parameters	Control	Phyto-biotic
Cecal tonsil	IL4	2.18±0.19[b]	7.29±0.47[a]
	IFN-γ	2.47±0.21[b]	5.59±0.26[a]
Spleen	IL4	3.27±0.51[b]	15.55±0.51[a]
	IFN-γ	3.77±0.5[b]	16.59±1.07[a]

Values are expressed as means ± standard error (SE); n=6. Means within the same row with different superscripts are significantly different (P<0.05).

DISCUSSION

In the present study, the Phyto-biotic showed a beneficial effect on the growth performance parameters including final body weight, total weight gain, and total feed efficiency as well as a significant reduction in total FCR than the basal diet-fed group. Such obtained results agreed with (Mohammadi et al., 2018). This positive effect of Phyto-biotic mainly due to its components that formed from a blend of essential oils as oregano oil, anise oil, and citrus oil (Hammer et al., 1999).

The essential oils have an appetizing effect as cause irritation of the taste buds present in buccal cavity leading to improvement of feed intake (Ertas et al., 2005) as well as these oils stimulate the nutrient digestibility via improving the digestive enzyme secretion and enhancing the utilization of digestive products through improvement of the liver functions (Al-Yasiry et al., 2018). Such materials have conventionally been used to enhance the release of endogenous secretions in the small intestine mucosa, pancreas and liver which in turn improve digestion and prevent adhesion of pathogens (Cross et al., 2007). The previous study by Zheng and co-workers clarified that the essential oils contributed to proper sedimentation of protein in broilers muscles Zheng et al. (2009).

Moreover, the essential oils create a fascinating environment inside the intestine due to its antimicrobials features especially oregano oil (Hammer et al., 1999). Together with the distinct effect of the essential oils on the intestinal morphological parameters via increasing villus height and villus height to crypt ratio and decreasing the crypt depth which directly correlated with proper absorption and positive performance responses (Awad et al., 2009a). In addition to, the antioxidant properties of essential oils, especially oregano, as contain abundant amounts of monoterpenes, thymol, and carvacrol which serve as absorbent to any feed toxins that possess a negative effect on the broilers health (Al-Yasiry et al., 2018). On the other hand, these results disagreed with Hernandez et al. (2004) who showed no effects of essential oils on broilers growth performance. The liver has a fundamental role in the metabolism, detoxification, and secretion of several enzymes and hormones. Moreover, the liver is the most sensitive organ to chemicals absorbed from the gut and the first organ affected by the toxic agents. When the plasma membrane of a hepatocyte is injured, a variety of the cytosolic enzymes will be released into the circulation, thus causing increased enzyme levels in the serum (Schmidt and Schmidt, 1983). Therefore, higher serum hepatic enzymes (AST and ALT) could be indicative of improper liver function due to inefficient performance (Khosravinia et al., 2013). Chickens fed on a diet supplemented with Phyto-biotic revealed a significant reduction ($P<0.05$) in serum ALT when compared with the control group at both 21 and 42 days of age. These results may have referred to the hepatoprotective effect of Phyto-biotic. This observation was supported by the findings of different researchers, who concluded that the essential oils possess a hepatoprotective effect in broiler chicks (Hernandez et al., 2004; Khosravinia et al., 2013; Al-Yasiry et al., 2018).

These essential oils mixture could possessed a biological activities such as antioxidant effects that enhance liver and kidney functions (Hernandez et al., 2004; El-Hack et al., 2018). Furthermore, Al-Yasiry et al. (2018) stated that the oregano oil, anise oil, and citrus oil combination had a hepatoprotective effect that showed a significant reduction in the levels of AST and ALT levels in broilers. The hepatoprotective effect of essential oils was attributed to their ability to repair hepatic cell injury and ability to lessen the pathogenic intestinal microbial loads. Therefore, they diminish the toxic metabolites and prevent enzyme leakage to the blood circulation (Hernandez et al., 2004). On contrary, Mansoub, (2011) reported that the oregano oil had no significant effects on blood biochemical parameters including the liver and kidney function. Moreover, Mostafa et al. (2016) revealed that the using of two diverse types of essential oils resulted in a negative effect on liver function.

Kidney are the second target organ that could be affected by the absorbed substances through the gut (Khosravinia et al., 2013). Uric acid level was significantly reduced by the addition of Phyto-biotic to the broiler diet when compared with the basal diet fed group at 21 days of the study. The results of this experiment announced the reno-protective activity of essential oils mixture which synchronized with the results obtained by (Eleiwa et al., 2011; Khosravinia et al., 2013) who revealed that the essential oils had a reno-protective effect in broiler chicks. In contrast, Mostafa et al. (2016) revealed that using two diverse types of essential oils resulted in a significant increase in the serum uric acid and creatinine levels.

The cellular, humoral and gut immunity is the local site of action of these additives. The cellular immunity (innate immunity) evaluation can be done by counting the number of macrophages in the blood that are able to engulf the pathogens (Al-Yasiry et al., 2018). The Phyto-biotic elicited a significant increase in the phagocytic percent and index than the control group which were similar to the results of Eleiwa et al. (2011). The essential oils improve the cellular immunity through stimulation of the complement receptor-mediated phagocytosis. Also, increasing the proportion cluster of differentiation of glycoprotein (CD4+ and CD8+) which found on the surface of immune cells and double the positive T cells in peripheral blood and mesenteric lymph nodes (Al-Yasiry et al., 2018). However,

Saleh et al. (2014) concluded that the essential oils had no distinct effect on phagocytic index and percent in broiler chicks.

In poultry, there are no lymph nodes while the lateral immune system comprises from spleen and gut-associated lymphoid tissue (GALT). The main GALT are payer's patches and cecal tonsils (Casteleyn et al., 2010). The GALT reacts with the microflora come from the feed and the environment. Thus, there was a relationship between the intestinal microflora and the gut associated lymphoid tissue (Bar-Shira et al., 2003). Therefore, the gastrointestinal community have evident effects on the gene expressions of the proinflammatory and anti-inflammatory cytokines and chemokines (Rakoff-Nahoum and Medzhitov , 2008). This study examined the effect of Phyto-biotic on the gene expression of IL4 (cytokine of th2) and IFN-γ (cytokine of th1) in both cecal tonsils and spleen at the age of 35 days of the study. The Phyto-biotic fed broilers exhibited a significant up-regulation of gene expressions of both IL4 and IFN-γ in cecal tonsils and spleen at the age of 35 days. These results were fit to the results obtained by (Paraskeuas et al., 2017) who proved that the essential oils have profound effects on the gene expressions of immune-related cytokines in the GALT of the broilers.

The estimation of the specific antibody titer against the viral vaccines has a prime interest in the poultry production nowadays because it gives an impression about the immune status of the flock andits resistance to the serious diseases especially those of viral causes (Zakeri and Kashefi, 2011). The current study showed that the antibody titer against ND and H5N1 were significantly increased in Phyto-biotic fed groups at 42 and 28 days, respectively than the control group. These results in accordance with the findings of (Talazadeh and Mayahi, 2017) who revealed that the essential oils showed a significant improvement in the humoral immunity against several viral vaccines. On contrary, Reza et al. (2013) demonstrated that the essential oils in the Phyto-biotic failed to create a significant improvement in the humoral immunity of broiler chicks.

The immune-stimulant effect of the Phyto-biotic used in this study could be mainly due to its components of essential oils mixture. These essential oils mixture pronounced effect on the broilers immune response could be through several factors as their capability to generate an optimum intestinal microbiota via enhancing the growth of beneficial bacteria in the intestine (Vidanarachchi et al., 2013), and diminishing the growth of pathogenic bacteria (Oussalah et al., 2007). That in contrast to antibiotics which inhibit the growth of both commensal and harmful bacteria in the intestine (El-Hack et al., 2018). Hence, Phyto-biotic not only stimulate the gut immunity through enhancement of the gut-associated lymphoid tissues which consider as a barrier against any ingested pathogens (Wagner, 2008), but also, stimulate the generalized immunity either cellular or humoral immunity. In conclusion, the Phyto-biotics addition in broilers diet resulted in a significant improvement in the growth performance, liver and kidney functions and immunity either local, cellular or humoral immunity. Future studies need to be directed toward the effect of Phyto-biotics on intestinal microbiome and metabolome.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest."None of the authors have any conflict of interest to declare".

AUTHORS CONTRIBUTION

Abdalim F. Abdalim and Mohamed K. Moursi conceived the original idea. Mohamed S. Helal and Eman Ahmed carried out the experiment. Mohamed S. Helal conducted all statistical analyses. Abdalim F. Abdalim supervised the project. Sawsan M. Elsheikh wrote the manuscript with support from Abdalim F. Abdalim and Eman Ahmed. All authors reviewed the final manuscript.

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