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Efficacy of phytase on the humoral immunity of broilers fed nutritionally marginal diets supplemented with citric acid and multi carbohydrase enzymes

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ABSTRACT

The aim of this study was to investigate the effects of using citric acid and multi carbohydrase enzymes on phytase efficacy on the humoral immunity of broiler chickens fed diets with nutritionally marginal calcium and nonphytate phosphorous (Ca-nPP). A total of 320 one-day-old male broiler chickens were randomly assigned to 8 treatment groups, with 4 replicates per treatment and 10 chicks per replicate in a completely randomized design. Eight dietary treatments containing, T1: adequate level of Ca-nPP as positive control diet (CTL+), T2: reduced levels of Ca-nPP as negative control (CTL−), T3: negative control diet + 1000 unit/kg phytase, T4: negative control diet + 1 % citric acid, T5: negative control diet + multi carbohydrase enzymes, T6: negative control diet + 1000 unit/kg phytase and 1 % citric acid, T7: negative control diet + 1000 unit/kg phytase and multi carbohydrase enzymes and T8: negative control diet + 1000 unit/kg phytase +1 % citric acid + multi carbohydrase enzymes. Blood samples were taken from each bird and the titers of maternal antibody against Newcastle disease virus (NDV) and avian influenza virus (AIV) were measured by haemagglutination-inhibition (HI) test. Addition of phytase to the diet increased significantly ($P<0.05$) antibody production against NDV and AVI as compared to birds fed CTL− diet in T2. In conclusion, the inclusion of citric acid and multi carbohydrase enzymes to the diet improved the effectiveness of phytase on antibody production against NDV and AVI in broiler chickens.

Key words: Phytase efficacy, broilers, citric acid, multi carbohydrase, humoral immunity.

INTRODUCTION

There are many of nutrients involved in the development of the immune response in poultry (Lesson, 2004). The efficacy of the immune system is potentially dependent on dietary situations, being adversely affected by an insufficient supply of either macronutrients (carbohydrates, fats, and proteins) or essential micronutrients such as vitamins, certain minerals, trace elements and essential amino acids (Klasing, 2007). It is well published that Ca-nPP in broiler diets impress growth, feed efficiency, bone development, leg health, nerve function and the immune system (Hassan and Al Aqil, 2015). The P demand for optimal immune response might be higher than for maximal growth (Aslam, 1995). Phytate is the main form of phosphorus that found in cereals, beans and oilseed meals feed to poultry (Ravindran et al, 1995).

Phytic acid is an strong chelating agent and forms a range of complexes with cations mainly the Ca^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} , Fe^{3+} and proteins, rendering these nutrients biologically unavailable (Ravindran, 1995). The common manner to resolve phytate problem is using microbial phytase enzyme. The beneficial effects of exogenous phytases in poultry ration has been reported to be due to the hydrolytic effect on phytate and the consequent enhancement in the availability of minerals, amino acids, and energy (Selle et al, 2007). In a theoretical stance, once phytic acid is

hydrolysed by phytase enzyme, all minerals bound to it should be released. The mineral-phytate complexes are usually formed at a pH that is above, or at the upper end of the activity spectrum of microbial phytase (Afsharmanesh and Pourreza, 2005). Hence, the prevalent pH in the gut could have a significant effect on the effectiveness of phytase. The bi-phasic pH profile of microbial phytase operation (Simons et al., 1990) indicates that subtle changes in pH of the upper digestive tract by inclusion of organic acids possibly will impress the activity of microbial enzyme. Besides, certain compounds with chelation ability (such as ascorbic acid and citric acid) have been shown to enhance mineral availability when involved in plant based diets fed to animals and possibly will operate as chelating agents (Afsharmanesh and Pourreza, 2005). Also, some factors including dietary supplementation with non-starch polysaccharide (NSP)-degrading enzymes, which have the potential to progress the release of P from phytate by phytase have been explored (Kim et al., 2005; Woyengo et al., 2008). NSP-degrading enzymes have been shown to improve nutrient consumption in poultry due to elimination of the nutrient encapsulating effect of cell walls and decline of digesta viscosity (Kim et al., 2005). Furthermore, NSP enzymes may also increase the efficacy of phytase by removing the phytate chelating effects of NSP (Kim et al., 2005). This is because NSP have the capability to bind multivalent cations (Debon and Tester, 2001), which associate with phytate in both feedstuffs and in digesta.

As a consequence, the effectiveness of microbial phytase may be enhanced by inclusion of organic acids and NSP-degrading carbohydrases to phytase-supplemented diet. Hence, the purpose of the current study was to assess the effects of citric acid, a multi carbohydrase and their combination on phytase enzyme efficiency on the humoral immunity of broilers fed nutritionally marginal diets.

MATERIALS AND METHODS

All experiments were carried out under the ethical guidelines of Payame noor University, Tehran, Iran (93/745, in 2014).

A total of 320, 1-day-old male broiler chicks (Ross308) were di-vided into 8 dietary treatments in a completely randomized design with 4 replicates of 10 birds each. Eight dietary treatments containing, T1: adequate level of Ca-nPP as positive control diet (CTL+), T2: reduced levels of Ca-nPP as negative control (CTL−), T3: negative control diet + 1000 unit/kg phytase (Natuphous10000; BASF Group, Ludwigshafen, Germany), T4: negative control diet + 1 % citric acid (Citric acid, Merck, Cat. No. 1.93427), T5: negative control diet + multi carbohydrase enzymes (Superzyme OM; Canadian Bio-Systems Inc.), which supplied 2,100 U of cellulase, 1,400 U of pectinase, 300 U of mannanase, 37.5 U of galactanase, 750 U of xylanase, 450 U of glucanase, 1,875 U of amylase, and 150 U of protease per kilogram of diet, T6: negative control diet + 1000 unit/kg phytase and 1 % citric acid, T7: negative control diet + 1000 unit/kg phytase and multi carbohydrase enzymes and T8: negative control diet + 1000 unit/kg phytase +1 % citric acid + multi carbohydrase enzymes. The chicks were raised on concrete floor pens covered with 8 cm of clean pine wood shavings, and each pen was equipped with a tube feeder and an automatic waterer. Throughout the study, the birds were brooded following standard temperature regimens, which gradually decreased from 32 to 23°C. Birds were maintained on a 23 L: 1D lighting schedule and allowed to consume feed and water *ad libitum*. Room temperature was kept according to the usual commercial practices. The birds were fed mash diets formulated according to Aviagen recommendations for Ross 308 broiler chickens (Table 1), (Aviagen, 2007). According to the producer company, the microbial phytase (Natuphos 10000 Granulate) contained 10,000 FTU/ g phytase activity. The enzymes (Natuphos 10000; BASF Group, Ludwigshafen, Germany and feedzyme 2000; granulate; UK) were added to the diets in powder form and all diets were fed as mash. Blood samples were taken from each bird and the titers of maternal antibody against Newcastle disease virus (NDV) and avian influenza virus (AIV) were measured by haemagglutination-inhibition (HI) test. At the age of 9 days, all chicks were vaccinated with Hitchner B1 NDV vaccine via eye (ophthalmic) route and bivalent killed vaccine (Newpasol 102, Inactivated W/O Emulsion ND + AI (H9N2) Vaccine, Pasouk Biological Co) by inoculation according of the manufacturer's recommendation. Blood samples were collected every week from the wing veins of individual chickens in all groups and their sera were separated and inactivated at 56°C for 30 min and kept at −20°C until analysis for the level of NDV and AVI antibodies. Serum antibody titer was measured by hemagglutination-inhibition test as described by Alexander et al. (1983) on days 7, 14, 21, 28, 35 and 42.

Statistical analysis

Data were analyzed by ANOVA in a completely randomized design, using the general linear models (GLM) procedure of SAS (SAS, 2008). Significant differences among treatment means were separated using duncan's multiple range test (Duncan, 1955). All statements of differences were based on significance at $P \leq 0.05$ and $P \leq 0.01$.

RESULTS

The effects of treatments on antibody production against NDV in broilers from day 7 to day 42 are presented in Table 2. On the 7th and 14th days of the study, there were no significant differences among antibody titers of experimental groups. Chickens of CTL− treatment showed reduction in antibody titers against NDV compared to the CTL+. Totally the addition of phytase to the diet increased antibody production against NDV in broilers compared to CTL−group. Birds fed the diet treated with citric acid and multi carbohydrase had higher antibody titers than others.

Also, the effects of treatments on antibody production against AVI in broilers from day 7 to day 42 are displayed in Table 3. On the 7th and 14th days of the examination, there was no significance alteration among antibody titers of experimental groups. As it showed, Chickens of CTL− treatment showed totally reduction in antibody titers against AVI virus compared to the CTL+. Moreover, these results demonstrated that positive effect of citric acid, multi carbohydrase enzymes and their combination on the phytase efficiency on the response to vaccination of the immune system of the chickens, fed diets with marginal amounts of calcium and phosphorous. Furthermore these consequences highlight again the positive effects of phytase addition in practical poultry rations.

DISCUSSION

Antibodies are essential biological mediators prevalent in the healthy immune repertoire, and they participate in the maintenance of immune homeostasis by exposure to environmental incitement (Bayry et al., 2005). It has been shown that low levels of humoral antibody may be related to disease susceptibility (Parmentier et al., 2004). Serum hemagglutination inhibition antibody is a valid index, since it is directly effective against NDV in the humoral immunity of broilers (Maas et al., 2003).

In the present study, compared to no treated marginal diets, the addition of phytase with citric acid and multi carbohydrase to the marginal diets, increased antibody production against NDV and AVI in broilers from 21 to 42 days of age. These results demonstrated that the positive influence of citric acid, multi carbohydrase enzymes and their combination on the phytase efficiency on the response to vaccination of the chicken's immune system. The existence of protein and phytate complexes have observed in most of grains. Rojas and Scott (1969) proposed that these complexes have adverse effects in poultry nutrition. It is recognized that phytate interacts with protein to form two different complexes depending on pH (Anderson, 1985). Selle et al. (2000) reported that increasing the digestibility of the proteins and amino acids by phytase is directly related to improve the immune response. Since immune cells are made of amino acids, so mentioned cells will help to improve immune responses in birds. In this study, intake of rations containing phytase enzymes resulted in higher titers, compared to birds fed diets without any treatments, specifically during the weeks in which titers tended to decrease. Consequently, phytate is a ubiquitous and strong antinutrient in monogastric diets and applies a variety of physiological, nutritional, and immunological consequences on the host. Compensatory mechanisms are in place to allow normal digestive processes to continue, but these carry a substantial nutritional cost to the animal in terms of energy, and amino acid and mineral requirements associated with synthesis, absorption, catabolism, and autolysis. An understanding of the antinutritive effects of phytate is an important first step in developing improved microbial phytases and in maximizing the potential of currently available phytase technology. Zyla et al (2000) reported that phytase addition to diets with a low P concentration enhanced the bursa weight of 21-days-old Hubbard broilers. Because the bursa is the source organ for B cells, the development of the bursa may induce the proliferation of B cells. Thus, the growth-promoting effect of phytase may be expressed via both nutrient release and a physiological regulation mechanism. The investigation of innate mucosal humoral immunity by Liu et al. (2008) showed that the levels of SIgA were increased by phytase addition. The mucosal epithelium is a potential effectors tissue of integrated host responses, producing SIgA to protect GI-associated port of entry into the body. The degradation products of phytate by phytase may regulate immune activity of these cells (Bozsik et al., 2007). Phytates may irritate the gut wall directly or by enhancing the growth of intestinal microflora, causing inflammation and provoking further immune response and increased production of cytokines (McKay and Baird, 1999).

Table 1. Composition of experimental diets[1]

	0 to 3 weeks								3 to 6 weeks							
Item	T1	T2	T3	T4	T5	T6	T7	T8	T1	T2	T3	T4	T5	T6	T7	T8
Ingredients (%of diet)																
Wheat	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Soybean meal	24.97	24.45	24.45	24.45	24.45	24.45	24.45	24.45	19.17	24.44	24.44	24.44	24.44	24.44	24.44	24.44
Corn	24.62	27.26	27.26	27.26	27.26	27.26	27.26	27.26	25.07	25.97	25.97	25.97	25.97	25.97	25.97	25.97
Corn Gluten	10	10	10	10	10	10	10	10	7.37	4.11	4.11	4.11	4.11	4.11	4.11	4.11
Wheat bran	-	-	-	-	-	-	-	-	7.21	5.54	5.54	5.54	5.54	5.54	5.54	5.54
Vegetable Oil	5.64	4.77	4.77	4.77	4.77	4.77	4.77	4.77	7	7	7	7	7	7	7	7
Di Calcium phosphate	1.75	0.92	0.92	0.92	0.92	0.92	0.92	0.92	1.3	0.67	0.67	0.67	0.67	0.67	0.67	0.67
Oyster shell	1.77	1.37	1.37	1.37	1.37	1.37	1.37	1.37	1.79	1.29	1.29	1.29	1.29	1.29	1.29	1.29
DL methionine	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.02	0.06	0.06	0.06	0.06	0.06	0.06	0.06
L lysine	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.28	0.14	0.14	0.14	0.14	0.14	0.14	0.14
Salt	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28
Vitamin premix[2]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mineral premix[3]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Citric acid	-	-	-	1	-	1	-	1	-	-	-	1	-	1	-	1
Phytase[4]	-	-	0.01	-	-	0.01	0.01	0.01	-	-	0.01	-	-	0.01	0.01	0.01
Multi Carbohydrase[5]	-	-	-	-	0.015	-	0.015	0.015	-	-	-	-	0.015	-	0.015	0.015
Nutrient composition																
CP (%)	23	23	23	23	23	23	23	23	20	20	20	20	20	20	20	20
ME (Kcal/Kg)	3200	3200	3200	3200	3200	3200	3200	3200	3200	3200	3200	3200	3200	3200	3200	3200
Lysine (%)	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1	1	1	1	1	1	1	1
Methionine (%)	0.523	0.523	0.523	0.523	0.523	0.523	0.523	0.523	0.386	0.392	0.392	0.392	0.392	0.392	0.392	0.392
Methionine + cysteine (%)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72
Tryptophan	0.223	0.223	0.223	0.223	0.223	0.223	0.223	0.223	0.204	0.214	0.214	0.214	0.214	0.214	0.214	0.214
Threonine (%)	0.771	0.770	0.770	0.770	0.770	0.770	0.770	0.770	0.667	0.670	0.670	0.670	0.670	0.670	0.670	0.670
Calcium (%)	1	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.9	0.63	0.63	0.63	0.63	0.63	0.63	0.63
Total Phosphorous (%)	0.703	0.550	0.550	0.550	0.550	0.550	0.550	0.550	0.410	0.309	309	309	309	309	309	309
Available phosphorous (%)	0.45	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.35	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Sodium (%)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15

[1]Calculated from NRC (1994). [2]Supplied per kilogram of diet: trans-retinyl acetate, 25 mg; cholecalciferol, 6 mg; menadione, 1.2 mg; thiamine, 2.3 mg; ribofavin, 8 mg; nicotinamide, 42 mg; choline chloride, 400 mg; calcium pantothenate, 10 mg; pyridoxine HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; cobalamin, 0.012 mg; Fe (from ferrous sulfate), 82 mg; Cu (from copper sulfate), 7.5 mg; Mn (from manganese sulfate), 110 mg; Zn (from zinc oxide), 64 mg; I (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.28 mg

However, activation of the immune system of the bird (e.g., increased production of antibodies in response of invading agents) is another energy-demanding process (Eraud et al., 2005). Gao et al. (2004) reported that supplementation of diets with nonstarch polysaccharide-degrading enzyme preparations significantly increased the anti-NDV titers of chicks. Kettunen and Rautonen (2005) reported that the use of xylanase, amylase, and protease or a combination of the enzymes and betaine enhanced nutrient uptake by intestinal cells and concluded that the concentration of IgA in the digesta contributed to improvements in immune competence. It might be due to changes in the viscosity of the diets or transit time through the digestive tract of the chicken. Phytase is able to release phytate-bound P and make further P available to the poultry. As phytase acts on the phosphate groups linked with the inositol ring of phytic acid backbone and thus releases P and Ca, it is predictable that the use of phytase would result in enhanced performance of the animal if P and Ca is the nutrient limiting for growth. Adeola and Bedford (2004) compared the efficacy of xylanase addition to high viscosity and low-viscosity wheat diets and reported that xylanase improved performance to a greater extent in high-viscosity compared with low-viscosity wheat, thus showing the relationship between the potential of a feed stuff to cause digesta viscosity and the beneficial effect that may result from enzyme usage.

Due to the use of corn-wheat -soybean meal diets that were marginally deficient in both P and Ca in the present study, calculation of enzyme produced a significant improvement in phytase efficacy as compared to the CTL−, demonstrating that P and Ca were limiting nutrients. As a result, the amounts of enzyme and inorganic P needed in diets could be minimized resulting in reduced cost, lower P excretion levels, and decreased environmental impact. Although a phytate molecule is a strong chelating agent, it is not the only molecule that is responsible for chelation

of nutrients. Parkkonen et al. (1997), using in vitro digestion techniques, reported that xylanase increased the permeability of the aleurone cell wall layer, which is the site of phytate storage. It is possible that xylanase, by improving aleurone layer permeability, enhances the access of endogenous phytase to phytate molecules, hence improving P digestibility and retention. It could be expected that an enzyme that is capable of breaking the NSP layer will also provide easier access to phytate. If phytase is used alone, the ability of the enzyme to act on phytate will be limited by its lack of access to its substrate if phytase is within the NSP matrix. Glycanase (xylanase and β-glucanase) that are able to break down the NSP fraction can facilitate the contact between phytase and phytate. Additionally, some soluble fiber bound P may be released in the presence of glycanase, and this may explain how glycanase is able to increase P digestibility.

Furthermore, the mineral-phytate complexes are usually formed at a pH that is above, or at the upper end of the activity spectrum of microbial phytase (Afsharmanesh and Pourreza, 2005). Therefore, the prevailing pH in the gut could have a significant result on the effectiveness of phytase. The bi-phasic pH profile of microbial phytase action (Simons et al., 1990) indicates that subtle alterations in pH of the upper digestive tract by adding of organic acids possibly will influence the activity of microbial enzyme. As well, citric acid have been shown to increase mineral availability when included in plant based diets fed to animals and may act as chelating agents (Afsharmanesh and Pourreza, 2005).

Table 2. Effect of citric acid and multi carbohydrase on phytase efficacy on Newcastle Disease Virus (NDV) antibody titers in broiler chicks from 7 to 42 days of age

	Antibody titers (day)					
Treatments	7th	14th	21st	28th	35th	42nd
T1	7.13	5.48	4.77 ^{a}	4.83 ^{ab}	5.41 ^{abc}	4.50 ^{ab}
T2	7.07	4.93	4.01 ^{b}	4.17 ^{b}	4.61 ^{c}	3.83 ^{b}
T3	6.83	5.20	4.40 ^{ab}	4.67 ^{ab}	5.53 ^{ab}	4.30 ^{ab}
T4	7.17	5.23	4.27 ^{ab}	4.70 ^{ab}	5.07 ^{abc}	4.33 ^{ab}
T5	6.67	4.98	4.26 ^{ab}	4.33 ^{b}	4.67 ^{bc}	4.17 ^{b}
T6	6.87	5.17	4.50 ^{ab}	5.09 ^{a}	5.47 ^{abc}	4.50 ^{ab}
T7	7.00	5.13	4.37 ^{ab}	4.87 ^{ab}	4.90 ^{bc}	4.33 ^{ab}
T8	7.17	5.37	4.67 ^{a}	5.25 ^{a}	5.80 ^{a}	5.13 ^{a}
	0.0356	0.0357	0.0471*	0.0702*	0.0849*	0.0725*

*$^{a–d}$ diferent superscript letters indicate a significant difference between data presented in the same row, * (P<0.05), ** (P<0.01)*

Table 3. Effect of citric acid and multi carbohydrase on phytase efficacy on Avian Flu Virus antibody titers in broiler chicks from 7 to 42 days of age

	Antibody titers (day)					
Treatments	7th	14th	21st	28th	35th	42nd
T1	7.00	4.67	3.43^{ab}	2.20 ^{bc}	2.47 ^{ab}	2.23 ^{ab}
T2	6.83	4.82	3.09 ^{b}	1.80 ^{c}	2.04 ^{b}	1.80 ^{b}
T3	7.10	4.70	3.87 ^{a}	2.47 ^{ab}	3.17 ^{ab}	3.00 ^{a}
T4	7.20	4.67	3.50^{ab}	2.17 ^{bc}	2.73 ^{ab}	2.48 ^{ab}
T5	6.80	4.52	3.17 ^{b}	2.23 ^{abc}	2.43 ^{ab}	2.37 ^{ab}
T6	6.83	4.67	3.83 ^{a}	2.67 ^{a}	3.23 ^{ab}	2.87 ^{a}
T7	7.13	4.97	3.33 ^{ab}	2.53 ^{ab}	3.43 ^{a}	2.57 ^{ab}
T8	6.93	5.07	3.67^{ab}	2.57 ^{ab}	3.27 ^{ab}	3.10 ^{a}
SEM	0.0323	0.0358	0.0566*	0.0561**	0.0975*	0.1038*

*$^{a–d}$ diferent superscript letters indicate a significant difference between data presented in the same row, * (P<0.05), ** (P<0.01)*

CONCLUSION

The results of current study indicated that citric acid and multi carbohydras enzymes affected the phytase efficacy which enhanced antibody production against NDV and AVI and consequently improved humoral immunity in broiler chickens, but these results did not obtain by adding of mentioned feed additives alone.

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