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Phytic Acid Anaylsis By Different Bacterial Phytases

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Abstract: This study aimed to measure the *in vivo* (using 70 male Cobb chicks experiments) degradation of the inositol hexaphosphate (IP_6) to lower insitol phosphate (IP2-IP5) using different phytases from *Aspergillus, Bacillus, E. coli and Klebsiella*. The broiler chicks experiment was designed according to the GFE (1999) including two basal diets, one formulated to contain adequate levels of all nutrients (diet A-positive control) while the other was deficient in the phosphorus supply (diet B-negative control) supplemented with the above mentioned enzymes. The inositol phosphates were analyzed in faeces and ileum content of all broiler chicks groups, the results revealed lower concentration of IP_2-IP_5 and higher concentration of IP_6 for all groups. Moreover, the inositol hexaphosphate (IP_6) degradation was determined, on the basis of daily intake and excretion of inositol hexaphosphate (IP_6). The IP_6 degradation was significantly increased by the supplementation of different phytases. The highest degradation rate was 8.5 ± 3.7 μM/g dry matter in faeces caused by the supplementation of *E. coli* and 7.8 ± 3.5 μM/g dry matter in ileum by *Bacillus* phytases.

Key words: Inositol phosphate, bacterial phytase, phytic acid.

INTRODUCTION

Phytic acid (PA) or Myo-inositol 1, 2, 3, 4, 5, 6 hexakisdihydrogen phosphate (IP_6) is widely distributed in nature. It is the principal form of phosphorus in many plants, ubiquitously distributed in plants, especially in mature legumes, cereal grains and oilseeds (Reddy *et al*., 1982; Oberleas, 1983). It is also found in roots, tuber and nuts (Marfo *et al*., 1990). It serves several physiological functions and also significantly influences the functional and nutritional properties of cereals, legumes and oilseeds, thereof by forming complexes with proteins and minerals. Phytic acid is composed of inositol (a polyalcohol) and six phosphate groups which are responsible for the powerful chelating ability of phytic acid (Oberleas, 1983). Therefore, phytic acid effectively binds different mono-, di- and trivalent cations and their mixtures, forming insoluble complexes (Reddy *et al*., 1989). The formation of insoluble phytate-mineral complexes in the intestinal tract of animals prevents mineral absorption. This reduces the bioavailability of essential minerals (Davies, 1982).

The enzyme phytase (myo-inositol hexakisphosphate phosphohydrolase) dephosphorylates phytic acid and/or phytate (myo-inositol hexakisphosphate) on successive steps terminating with formation of lower myo-inositol phosphate and free orthophosphate and in some cases o free myo-inositol and, thereby destroys the chelating ability of phytic acid (Eskin and Wiebe, 1983). Phytase supplementation as feed additives, which degrades phytate to release phosphorus and other nutrients, has been very beneficial in solving partially the aforementioned problems as shown by several previous studies (Boling *et al*., 2000; Jalal and Scheideler, 2001; Igbasan *et al*., 2001; Ceylan *et al*., 2003; Elkhalil, *et al*., 2007).

Many attempts have studied the path way of degradation of phytate through HPLC techniques. Greiner *et al*. (1993) have studied the pathway of *E. coli* phytase and found that *E. coli* phytase is able to hydrolyse five phosphate groups from phytic acid ring. Furthermore, Sajidan (2002) has found similar results for *K. pneumoniae* but in a different pathway. Kerovuo *et al*. (2000) have studied the path way of hydrolysis of phytic acid by *Bacillus* phytase and results revealed that the enzyme hydrolyses only three phosphates groups from phytic acid. Moreover, the enzyme seems to prefer the hydrolysis of every second phosphate over that of adjacent ones. Furthermore, it is very likely that the enzyme has two alternative pathways for the hydrolysis of phytic acid, resolution to different myo-inositol trisphosphate end products: Ins(2,4,6)P_3 and Ins(1,3,5)P_3. The objectives of this study were to determine the concentration of the different inositol phosphate isomers in the ileum contents and faeces of broiler chicks after degradation of phytic acid by different bacterial phytase enzymes and to calculate the percentage of phytic acid degradation products.

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MATERIALS AND METHOD

Enzymes:

Three bacterial phytases were used in this study and compared with a commercial *Aspergillus* phytase (Natuphos, 5000G, BASF). The bacterial phytases were an *E. coli* phytase produced at a pilot scale. Another two bacterial phytases were produced at a laboratory scale. One was a *Klebsiella* phytase which was produced as and the other was a *Bacillus* phytase produced using the *Bacillus amyloliquefaciens* strain DSM 7. The enzymes used in this study were produced as de described by Elkhalil *et al.* (2007). The enzyme activity was measured using method of Engelen *et al.* (1994).

Animal Experiments:

A total of 70 male Cobb chicks were used in the trial. They received from day one to 14 post-hatching, an adequate diet with regard to all nutrient supply according to the recommendations of the GFE (1999). Then the animals were transferred to metabolic cages and divided at an equal number (10 birds) to one of seven dietary treatments. The basal diets where formulated to contain adequate levels of all nutrients (diet A – positive control) or to be deficient in the phosphorus supply (diet B – negative control). Both diets are based on corn and soya bean meal, contained metabolizable energy 12.7 MJ/kg, crude protein 185.5 g/kg, calcium 8.2 g/kg and available phosphorus 4.6 and 3.1 g/kg, respectively. The other treatments were diet B supplemented with the various enzymes as indicated in Table 1. For balance assay, birds were fed the experimental diets for five days (adaptation period) prior to 3 d collection period. During this period feed intake was measured and all excreta voided were collected and stored in -20°C, frozen excreta samples were freeze-dried, weighed, finally ground and pooled for chemical analysis. Also, the samples were taken from the ileum content.

Table 1: Diet treatment containing Phytase activity.

Treatment	Specification	Expected Phytase activity (FTU/kg)	Estimated Phytase activity (FTU/kg)
A	Positive-P-control	-	62
B	Negative-P-control	-	59
C	As B+ *Bacillus* phytase	700	741
D	As B+ *Klebsiella* phytase	500	563
E	As B+ *E. coli* phytase	500	561
F	As B+(Bacillus 350 + E. coli 250) phytase	600	647
G	As B + *Aspergillus* phytase	500	442

Table 2: Effects of different phytases on the inositol hexaphosphate degradation[1] [%] during passage the digestive tract of broiler chicken.

group	specification	IP_6 intake [g/3 d]	IP_6 excretion [g/3 d]	Degradation [%]
A	Positive-P-control	0.79 ± 0.19	0.51 ± 0.16	35.51 ± 12.71^{a}
B	Negative-P-control	0.84 ± 0.14	0.56 ± 0.07	32.54 ± 5.72^{a}
C	As B+ *Bacillus* phytase	0.74 ± 0.11	0.21 ± 0.10	72.36 ± 10.32^{bc}
D	As B+ *Klebsiella* phytase	0.69 ± 0.09	0.28 ± 0.11	58.64 ± 18.04^{b}
E	As B+ *E. coli* phytase	0.71 ± 0.19	0.15 ± 0.10	77.50 ± 16.19^{c}
F	As B+(*Bacillus* 350 + *E. coli* 250) phytase	0.61 ± 0.12	0.24 ± 0.08	61.41 ± 9.92^{b}
G	As B + *Aspergillus* phytase	0.78 ± 0.29	0.45 ± 0.19	43.14 ± 16.37^{a}

1. Determined on the basis of a balance study over 3 days and calculated as the difference between the mean daily intake and excretion of the inositol hexaphosphate per chicks.

* Data are means of ten chicks; data were analyzed by one way ANOVA using Duncan's test of SPSS. (SPSS, 1999, version 10.0) at ($P < 0.05$) level of significant.

Chemical Analysis:

Analysis of the Inositol Phosphates:

Sample Preparation:

The sample preparations were performed according to the method of Sandberg and Ahderinne (1986), in the manner described by Skoglund *et al.* (1997) with a minor modification. Sample of 0.25-0.5 g of freeze dried feedstuffs, intestinal content or faeces collection were extracted with 20 ml 0.5M HCl for 3 h at room temperature under agitation. The extracted samples were centrifuged for 15 min at 2600 x g and the supernatant decanted. Then the supernatant was filtered (Schleicher and Schuell filter paper No. $595^{1}/_{2}$, Dassel, Germany). Fifteen milliliters of the filtered supernatant were taken out and frozen overnight and then freeze dried to dryness and dissolved in 15 ml of water. The Inositol phosphates were separated from the crude extract by ion exchange chromatography; SPE columns with porous polymer filter containing 2.5 ml of resin (AG 1-X8, 200-400 mesh, Bio-Rad, Hercules, Canada) were used. The samples were washed twice with 5 and 10 ml water and inositol phosphates were removed from the resin with four 5 ml portions of 2M HCl. The eluants were freeze dried to dryness and diluted with 1 or 2 ml of water.

A reference sample for identification of peaks was prepared by dissolving 1.5 g of sodium phytate in 100 ml of 0.5 M HCl. The solution was reflux boiled for 12 h and evaporated to dryness and 100 ml of water were added to the hydrolyzed sample.

HPLC Analysis of The Inositol Phosphate Isomers:

Inositol phosphates (IP2-IP6), 100µl were separated on Mono-Q using short column (5 cm; HR 5/5, Pharmacia), good separation of different inositol phosphate isomers in a short run (32 min) was achieved. Inert HPLC system was applied (Gradient Pump 2249 and low Pressure Mixer; Pharmacia) and a gradient of HCl used (0.005 - 0.5mol/L; flow rate: 1 ml /min). To improve separation of inositol phosphate isomers and to protect the column, a layer of Source 20 (1cm) (Pharmacia) was placed on top of the Mono-Q creating a one-column system. Post-column derivatisation was obtained by mixing the eluent with 0.1mol/L HCl, containing 5.18mmol/l FeCl3 and 0.25 mol/l NaCl, via a twisted PTFE coil (length 10 m; i.d. 0.5 mm) (HPLC pump 2248, Pharmacia; flow rate: 0.8 ml/min). Absorption was measured at 290 nm (UV-Vis detector VWM2141, Pharmacia). Peaks were identified by standard addition of inositol phosphate standard. Chromatograms were evaluated by the PC Integration Pack software (Kontron-Biotek, Neufahrn, Germany).

Statistical Analysis:

Analysis of variance (ANOVA) was performed on one-way ANOVA using the Duncan multiple range test to separate means procedure of SPSS (SPSS, 1999, ver. 10.0) appropriate for a completely randomized design. Significance was accepted at $P \leq 0.05$.

RESULTS AND DISCUSSION

Analysis of The Inositol Phosphates:

Inositol Phosphates In Faeces:

The analysis of the composition of inositol phosphates of the faeces contents of broiler chicks revealed higher concentration of inositol hexaphosphate (IP_6) (Fig 1) and lower concentration of the sum of inositol diphosphate (IP_2), inositol triphosphate (IP_3), inositol tetraphosphate (IP_4) and inositol heptaphosphate (IP_5) (Fig 2) for all broiler treatments. The inositol monophosphate (IP_1) was not detected due to technical difficulties.

For positive control diet (A), higher concentration of IP_6 was detected (19.4 ± 1.5 µm/g dry matter), which was not different ($P < 0.05$) (Fig. 1) from the negative control diet (B; 21.5 ± 1.9 µm/g dry matter). The concentration of sum of IP_2-IP_5 was found for both positive and negative control diets as 5.8 ± 1.8, 5.3 ± 0.9 µM/g dry matters, respectively (Fig. 2). For groups D, E and F where *Klebsiella* phytase, *E. coli* phytase and the combination of *E. coli* with *Bacillus* phytases were supplemented to the P-deficient diet, IP_6 was significantly ($P < 0.05$) reduced compared to both control diet, amounting 14.0 ± 6.5, 9.3 ± 4.9 and 13.8 ± 3.5 µM/g dry matter, respectively. The highest reduction of IP_6 caused by supplementation of *Bacillus* phytase to negative control diet was 8.5 ± 3.7 µM/g dry matters, however it was not significantly different ($P < 0.05$) from that caused by *E .coli* phytase. In contrast to bacterial phytases, the *Aspergillus* phytase caused not significant ($P < 0.05$) reduction of IP_6 (18.6 ± 5.3 µM/g dry matters) to the both control diets when supplemented to P-deficient diet (group G). But at the same time, it was not significantly different from the reduction caused by *Klebsiella* phytase (D) and the combination of *E. coli* and *Bacillus* phytases (F).

The concentration of IP_2-IP_5 was significantly ($P < 0.05$) lower for group D, E, F and G compared to both control diets as shown in Figure 2.

Figure 3 shows the concentration of total inositol phosphates (IPs) in faeces content. The total IPs were significantly ($P < 0.05$) reduced by supplementation of all phytases. The lowest reduction was caused by *Aspergillus* phytase while the highest reduction was caused by *E. coli* phytase.

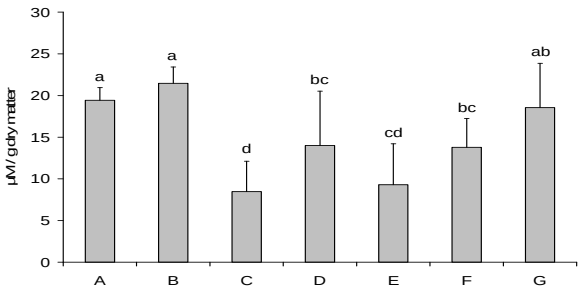

Fig. 1: Effect of different phytases on the concentration of IP_6 in faeces of broiler chicken.

A. +ve- control (diet with adequate phosphorus supply).
B. -ve control (diet with deficient phosphorus supply).
C. As B + *Bacillus* phytase (700 FTU/kg).
D As B + *Klebsiella* phytase (500 FTU/kg).
E. As B + *E. coli* phytase (500 FTU/kg).
F. As B + (*E. coli* + *Bacillus*) phytase (250 +350 FTU/kg).
G. As B + *Aspergillus* phytase (500 FTU/kg).
* Data are means of ten chicks; data were analyzed by one way ANOVA using Duncan's test of SPSS. (SPSS, 1999, version 10.0) at ($P < 0.05$) level of significant.

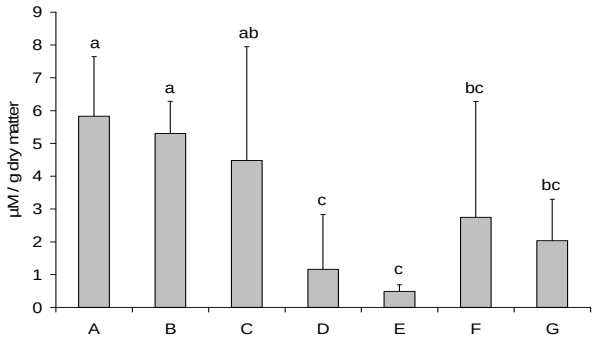

Fig. 2: Effect of different phytases on the concentration of IP_2-IP_5 in faeces of broiler chicken.

A. +ve- control (diet with adequate phosphorus supply).
B. -ve control (diet with deficient phosphorus supply).
C. As B + *Bacillus* phytase (700 FTU/kg).
D As B + *Klebsiella* phytase (500 FTU/kg).
E. As B + *E. coli* phytase (500 FTU/kg).
F. As B + (*E. coli* + *Bacillus*) phytase (250 +350 FTU/kg).
G. As B + *Aspergillus* phytase (500 FTU/kg).
* Data are means of ten chicks; data were analyzed by one way ANOVA using Duncan's test of SPSS. (SPSS, 1999, version 10.0) at ($P < 0.05$) level of significant.

Inositol Phosphates In Ileum Content:

The analysis of inositol phosphates of ileum content also indicated higher concentration of IP_6 (Fig.4) and lower concentration of IP_2-IP_5 (Fig. 5). The concentration of IP_6 of group D (*Klebsiella* phytase) was significantly higher ($P < 0.05$) compared to the other treatments. While groups (F and G), where combination of (E. coli and Bacillus; group F) and Aspergillus (group G) phytases were supplemented to negative control diet were not different from both control diets (Fig. 4). The lowest concentration of IP_2-IP_5 was detected in group E (*E. coli* phytase) while no IP_2-IP_5 was detected in treatment D (Fig. 5).

Figure 6 shows the total concentration of inositol phosphate in ileum contents. Supplementation of *Bacillus, E. coli* and *Aspergillus* phytases and the combination of *Bacillus* with *E. coli* phytases did not affect the total inositol phosphates. *Klebsiella* phytase had also no affect on total IPs; however, it was significantly ($P < 0.05$) higher from the both control diets.

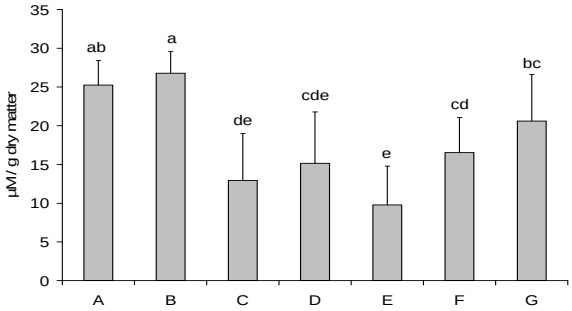

Fig. 3: Effect of different phytases on the total concentration of IPs in faeces of broiler chicken.

A. +ve- control (diet with adequate phosphorus supply).
B. -ve control (diet with deficient phosphorus supply).
C. As B + *Bacillus* phytase (700 FTU/kg).
D As B + *Klebsiella* phytase (500 FTU/kg).
E. As B+ *E. coli* phytase (500 FTU/kg).
F. As B + (*E. coli* + *Bacillus*) phytase (250 +350 FTU/kg).
G. As B + *Aspergillus* phytase (500 FTU/kg).
* Data are means of ten chicks; data were analyzed by one way ANOVA using Duncan's test of SPSS. (SPSS, 1999, version 10.0) at ($P < 0.05$) level of significant.

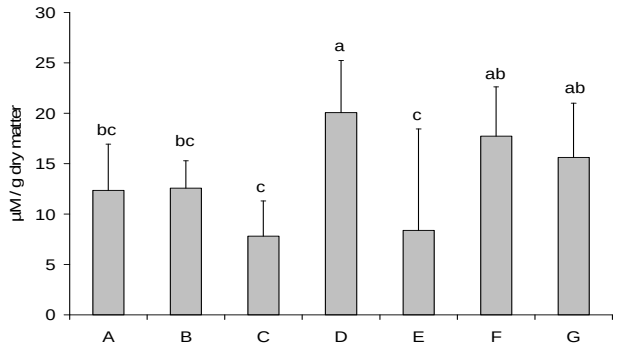

Fig. 4: Effect of different phytases on the concentration of IP_6 in ileum of broiler chicken.

A. +ve- control (diet with adequate phosphorus supply).
B. -ve control (diet with deficient phosphorus supply).
C. As B+ *Bacillus* phytase (700 FTU/kg).
D As B+ *Klebsiella* phytase (500 FTU/kg).
E. As B + *E. coli* phytase (500 FTU/kg).
F. As B+ (*E. coli* + *Bacillus*) phytase (250 +350 FTU/kg).
G. As B + *Aspergillus* phytase (500 FTU/kg).
* Data are means of ten chicks; data were analyzed by one way ANOVA using Duncan's test of SPSS. (SPSS, 1999, version 10.0) at ($P < 0.05$) level of significant.

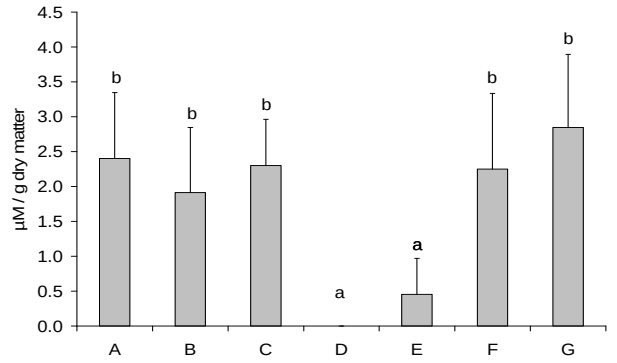

Fig. 5: Effect of different phytases on the concentration of IP_2-IP_5 in ileum of broiler chicken.

A. +ve- control (diet with adequate phosphorus supply).
B. -ve control (diet with deficient phosphorus supply).
C. As B + *Bacillus* phytase (700 FTU/kg).
D As B + *Klebsiella* phytase (500 FTU/kg).
E. As B + *E. coli* phytase (500 FTU/kg).
F. As B + (*E. coli* + *Bacillus*) phytase (250 +350 FTU/kg).
G. As B + *Aspergillus* phytase (500 FTU/kg).
* Data are means of ten chicks; data were analyzed by one way ANOVA using Duncan's test of SPSS. (SPSS, 1999, version 10.0) at ($P < 0.05$) level of significant.

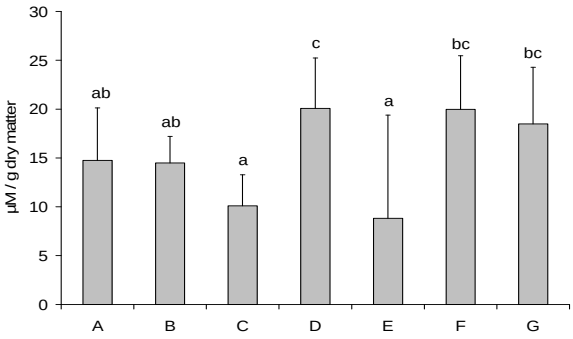

Fig. 6: Effect of different phytases on the total concentration of IPs in ileum of broiler chicken.

A. +ve- control (diet with adequate phosphorus supply).
B. -ve control (diet with deficient phosphorus supply).
C. As B + *Bacillus* phytase (700 FTU/kg).
D As B + *Klebsiella* phytase (500 FTU/kg).
E. As B + *E. coli* phytase (500 FTU/kg).
F. As B+ (*E. coli* + *Bacillus*) phytase (250 +350 FTU/kg).
G. As B + *Aspergillus* phytase (500 FTU/kg).
***** Data are means of ten chicks; data were analyzed by one way ANOVA using Duncan's test of SPSS. (SPSS, 1999, version 10.0) at ($P < 0.05$) level of significant.

Balance Study For Inositol Hexaphosphate:

On the basis of the mean daily intake and excretion of inositol hexaphosphate (IP_6) per chicks, the mean degradation of inositol hexaphosphate (IP_6) during the passage through the digestive tract was calculated (Table 2). IP_6 balance studies revealed that IP_6 degradation was similar for the phosphorus-adequate diet (positive control) and for the phosphorus-deficient diet (negative control), amounting to 35.5 and 32.5%, respectively. Addition of the Aspergillus phytase to the phosphorus-deficient diet, IP_6 caused no significant degradation (43.1%) compared to both control diets. For treatments C, D and F where *Bacillus* phytase, *Klebsiella* phytase and the combination of *Bacillus* and *E. coli* phytases were supplemented to the phosphorus-deficient control diet, IP_6 degradation was significantly ($P < 0.05$) increased amounting to 72.3, 58.6 and 61.1%, respectively. The IP_6 degradation was highest in treatment E (77.5%) where E. coli phytase was supplemented. However, this effect was not significant different from treatment C where *Bacillus* phytase was supplemented to phosphorus-deficient diet.

Discussion:

The analysis of the composition of inositol phosphates in ileum content and faeces revealed lower concentration of IP_2-IP_5 and higher concentration of IP_6. This resulted from the increased degradation of inositol phosphates followed by higher absorption leading to higher concentration of non absorbable components. High concentration of IP_6 and low concentration of IP_2-IP_5 showed preferred hydrolysis of the lower inositol phosphates than phytate (Schlemmer *et al.*, 2001). This was in agreement with Jany *et al.* (1999) who found *in vitro* strong degradation of inositol pentaphosphates by alkaline phosphatases. For this reason high activity of alkaline phosphatases and low activity of phytases in the small intestine and colon may explain why the hydrolysis of lower inositol phosphates is preferred than phytate, with exception of *Bacillus* phytase, which preferred to be active at those segments of the digestive tract (pH optimum; 7.0-7.5). *Bacillus* phytase (as well as *E. coli* phytase) caused the highest IP_6 reduction among the other phytases in ileum and faeces amounting to 8.5 ± 3.7 µM/g dry matter in faeces and 7.8 ± 3.5 µM/g dry matter in ileum (Figures 1 and 4). They also caused the highest IPs reduction (Figures 3 and 6).

The analysis of inositol phosphates in ileum reflected no obvious result. However, that was not expected due to the sampling procedure, the sample was collected after the slaughtering the animals from content of ileum (Meckek's diverticulum to ileo-cecal) as spot sample weighed not more than two grams.

Inositol phosphates degradation during the passage through the digestive tract of broiler chicken was significantly different between the groups due to the different phytases and their different hydrolysis pathways. The highest phytate degradation was caused by the supplementation of *E. coli* phytase. This result confirms that *E. coli* phytase was more resistant to protease inactivation and was more stable during passage through the different segments of digestive tract. Moreover, that the *E. coli* phytase has the ability to hydrolyze five phosphorus molecules from phytic acid (Greiner *et al*., 1993). All these lead *E. coli* phytase to be superior to the

other phytases used in this study. At the same time, the phytate degradation caused by *E. coli* phytase was not different (P < 0.05) from that caused by *Bacillus* phytase. This result could be attributed to *Bacillus* phytase which has the ability to be active in the lower part of digestive tract due to its pH optimum.

The degradation of phytate caused by treatment F where *E. coli* phytase was combined with *Bacillus* phytase, was not different (P <0.05) from that caused by *Bacillus* phytase but was different (P <0.05) from that caused by *E. coli* phytase. This result could be explained due using half the amount of the both of *E. coli* and *Bacillus* phytases and also could be attributed to the lower feed intake for this treatment (155 g/ 3 day).

The lowest inositol phosphate degradation caused by the *Aspergillus* phytase, which was not significantly different from both control diets. That is may have been due to the *Aspergillus* phytase not being stable during passage through the digestive tract and was susceptible to proteases inactivation that led *Aspergillus* phytase to be active only at the upper part of digestive tract during a limited time period.

Only a few direct estimations of the phytate degradation by phytase in broiler chicken have been reported. Camden *et al*. (2001) found that a phytase feed enzyme degraded 23.1, 26.3 and 31.6% of phytate in a maize-soybean meal diet in ileum at 250, 500 and 1000 FTU/kg, respectively. While Tamim *et al*. (2004) reported that phytase at 500 FTU/kg hydrolyzed 33.5% of dietary phytate in a maize-soybean meal diet with addition of the calcium.

Conclusion:

It could be concluded that the *E. coli* and *Bacillus* phytases and their combination improved the inositol phosphate degradation most effectively. This work indicates that phytate degradation by those enzymes seems worth to be followed in future research to increase the availability of phytate phosphorus.

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