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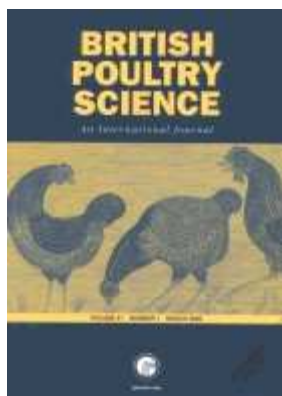
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THE EFFECT OF CITRIC ACID AND MICROBIAL PHYTASE ON AMINO ACID DIGESTIBILITY IN BROILER CHICKENS

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14 7 **Effects of citric acid and microbial phytase on amino acid digestibility in broiler**
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16 8 **chickens**
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4 36 **Abstract** 1. Two experiments with growing chickens were carried out to study the
5
6 37 effects of the inclusion of a microbial phytase (Natuphos[®] 5000) and citric acid (CA) in
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8 38 maize-soybean based diets on the performance and apparent ileal digestibility (AID) of
9
10 39 crude protein (CP) and amino acids (AA). In both experiments the diets were
11
12 40 formulated to contain the same amounts of energy and protein.

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14
15 41 2. In the first experiment, data were analysed as a 2 x 2 factorial arrangement with two
16
17 42 concentrations of available phosphorus (AP) from 1 d to 3 weeks of age (3.5 and 2.2
18
19 43 g/kg) and for 3 to 6 weeks (2.7 and 1.4 g/kg), and two inclusions of commercial phytase
20
21 44 (0 and 500 FTU/kg) in each period. The AID of CP and dispensable and indispensable
22
23 45 AA were not modified by the AP content of the diet. Addition of phytase improved the
24
25 46 AID of CP and dispensable and indispensable AA only at low AP levels.

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29 47 3. In the second experiment, data were analysed as a 3 x 2 factorial arrangement with
30
31 48 three concentrations of citric acid (0, 20 and 50 g/kg) and two inclusions of commercial
32
33 49 phytase (0 and 750 FTU/kg). Diets were formulated with deficient contents of AP (2.5
34
35 50 g/kg). Performance was not affected by commercial phytase addition. The addition of
36
37 51 CA reduced the weight gain but did not modify the feed intake and gain:feed. In
38
39 52 general, the AID of CP and dispensable and indispensable AA were not affected by CA
40
41 53 addition. Commercial phytase increased the apparent ileal digestibility of crude protein
42
43 54 but had no effect on AID of dispensable and indispensable AA.

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48 55 4. In conclusion, the present work showed that microbial phytase enhanced AA
49
50 56 digestibility in maize-soy-based diet only at very low AP concentrations, and that CA
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52 57 had no affect on the AID of CP and dispensable and indispensable AA. No synergism
53
54 58 between CA and microbial phytase was detected.
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INTRODUCTION

The capacity of phytic acid to bind minerals reduces the digestion and absorption of phosphorus, calcium, magnesium and zinc from plant-derived ingredients by monogastric animals (Jonbloed *et al.*, 1996; Ravindran *et al.*, 1999; Viveros *et al.*, 2002). Many reports have indicated that the addition of microbial phytase to poultry diets can release inorganic phosphate from the phytate complex, thus improving phosphorus availability, and thereby reducing phosphorus excretion (Ravindran *et al.*, 2000; Viveros *et al.*, 2002). The effect of phytate on the utilisation by poultry and swine of nutrients other than P, however, has received little attention until recently. The basis for these effects probably lies in the strong chelating potential of phytic acid by its ability to form protein complexes (Gifford and Clydesdale, 1990; Selle *et al.*, 2000), by its phytate-induced increases in endogenous amino acid flows (Ravindran *et al.*, 1999; Cowieson *et al.*, 2004) and by the inhibition or reduction of trypsin enzyme (Caldwell, 1992). The literature on phytate-mediated improvements in protein utilisation represents a conflicting base of information. The efficacy of phytase in improving protein utilisation has been inconsistent, data previously reported in several studies having suggested that microbial phytase can have beneficial effects on amino acid digestibility in individual feed ingredients (Ravindran *et al.*, 1999) and in compound diets (Yi *et al.*, 1996; Ravindran *et al.*, 2000; Rutherfurd *et al.*, 2002, 2004; Ravindran *et al.*, 2006), whereas other investigators have not obtained statistically significant responses (Biehl and Baker, 1997; Peter and Baker, 2001; Auspurger and Baker, 2004; Onyango *et al.*, 2005).

Citric acid (CA) has been reported to intensify phytate dephosphorylation *in vitro* (Zyla *et al.*, 1995). More recent research has indicated that citric acid is also very efficacious in improving P utilisation in chickens fed on maize-soybean meal diets

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3 84 containing no supplemental P (Boling *et al.*, 2000; Brenes *et al.*, 2003; Snow *et al.*,
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5 85 2004; Rafacz-Livingston *et al.*, 2005) and in one study reduced the amount of needed
6
7 86 supplemental phosphorus by approximately 0.1% of the diet (Boling-Frankenbach *et al.*,
8
9 87 2001). In contrast, citric acid does not improve the utilisation of dietary P in laying hens
10
11 88 given diets containing 38 g/kg Ca (Boling *et al.*, 2000; Snow *et al.*, 2004). There has
12
13 89 been recent interest in combining citric acid and phytase to investigate possible
14
15 90 interactive effects in poultry. Brenes *et al.* (2003) reported that the combination of
16
17 91 phytase and citric acid did not improve phosphorus and calcium availability above that
18
19 92 obtained with phytase alone. Since there is no clear consensus on the effects of
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21 93 microbial phytase addition on protein and amino acid digestibility in maize-soybean
22
23 94 diets, and even less regarding the efficiency of phytase and citric acid on this
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25 95 measurement, the objective of the current study was to determine the effects of phytase
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27 96 and citric acid on the apparent amino acid digestibility in a maize-soybean diet fed to
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29 97 broiler chickens.

98 MATERIALS AND METHODS

99 **General experimental procedures**

100 Male chicks (Cobb) were obtained from a commercial hatchery and used in all
101 experiments. Chicks were housed in electrically heated stainless steel battery brooders
102 in an environmentally controlled room with 23-h constant overhead fluorescent lighting.
103 Diets in pellet form (Experiment 1) and in mash form (Experiment 2) and water were
104 provided for *ad libitum* consumption. Celite, a source of acid-insoluble ash (AIA) was
105 added at 10 g/kg to all diets as an indigestible marker. Mortalities were recorded daily.
106 At the end of each experimental period (6 weeks, Experiment 1 and 3 weeks,
107 Experiment 2), birds were weighed and feed consumption was recorded for calculation
108 of gain:feed ratio. Microbial phytase (Natuphos[®] 5000, BASF, Mount Olive, NJ 07828-

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3 109 1234, USA) containing 5,000 phytase units (FTU)/g phytase activity was used in both
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6 110 experiments. One FTU is the quantity of enzyme that releases 1 μmol of inorganic
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8 111 P/min from 0.00015 mol/L sodium phytate at pH 5.5 at 37 °C. Analysed dietary amino
9
10 112 acids are shown in Table 2. All housing and handling were approved by the University
11
12 113 Complutense of Madrid Animal Care and Ethics Committee in compliance with the
13
14 114 Ministry of Agriculture, Fishery and Food for the Care and Use of Animals for Scientific
15
16 115 Purposes.

116 **Experiment 1**

117 The objective of this experiment was to evaluate the effects of two available phosphorus
118 (AP) concentrations and two inclusions of supplemental phytase on the apparent ileal
119 protein and amino acid digestibilities. A total of 240 male 1-d-old broiler chickens were
120 allocated to 30 pens, each pen containing eight chicks, to receive five dietary treatments
121 of maize-soybean-based diets (Table 1) with six replicates of each treatment. At the end
122 of 3 weeks, birds were moved from starter to grower finisher batteries for the remaining
123 3 weeks.

124 The experimental design was a 2 x 2 factorial with an additional diet (control
125 diet) with two levels of phytase (0 and 500 FTU/kg) and 2 concentrations of AP
126 (control minus 0.1 % and control minus 0.23 %) at each two phases (starter and
127 grower). AP and Ca concentration were 4.5 and 9.9 g/kg (starter) and 3.7 and 8.7 g/kg
128 (grower-finisher) in the control diet. The concentration of Ca was left the same when
129 AP was changed. The Ca:tP ratio of the control, T₂-T₄ and T₃-T₅ was 1.39, 1.62 and
130 2.02 in the starter diet, and 1.40, 1.67 and 2.20 in the grower-finisher diet, respectively. Table 2 near here

131 **Experiment 2**

132 The objective of this experiment was to determine the effect of citric acid alone or in
133 combination with phytase on performance and apparent ileal protein and amino acid

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3 134 digestibilities in P-deficient diets (2.5 g/kg AP). A total of 288 male 1-d-old broiler
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5 135 chickens were allocated to 36 pens, each pen containing 8 chicks, to receive 6 dietary
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7
8 136 treatments of maize-soybean-based diets (Table 1) with 6 replicates of each treatment.
9

10 137 The experiment consisted of a 3 x 2 factorial arrangement of the treatments with
11
12 138 3 concentrations of citric acid (Sigma-Aldrich Quimica, Tres Cantos, Spain) (0, 20 and
13
14
15 139 50g/kg) and two levels of supplemental phytase (0 and 750 FTU phytase/kg diet).
16
17 140 Proportions of maize, soybean meal and sunflower oil were modified in diets to
18
19 141 maintain ME (13.0 MJ/kg) and crude protein (230 g/kg) constant. The ME of citric acid
20
21 142 was assumed to be 10.3 MJ/kg (FEDNA Tables). In both experiments, dietary
22
23 143 concentrations were formulated to be below the current NRC (1994) to ensure
24
25 144 maximum responses with phytase.
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29 145 **Collection of samples and measurements**

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31 146 At the end of the experimental period (6 weeks, Experiment 1 and 3 weeks, Experiment
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33 147 2), 12 birds per treatment were weighed and sacrificed by cervical dislocation. The
34
35 148 ileum was dissected within 5 min after killing. The ileum was defined as that portion of
36
37 149 the small intestine extending from Meckel's diverticulum to a point of 40 mm proximal
38
39 150 to the ileo-caecal junction. Ileal digesta of birds within a pen were pooled, resulting in 6
40
41 151 composite samples per dietary treatment. The digesta were frozen immediately after
42
43 152 collection and subsequently freeze-dried. Digesta samples were ground to pass through
44
45 153 a 0.5-mm sieve and stored in airtight containers at -4 C for chemical analyses.
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50 154 **Chemical analyses**

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3 155 Diets and ileal contents were analysed for crude protein (6.25 N) using a standard
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5 156 method (Association of Official Analytical Chemist, 1990). The AIA contents of diet
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7 157 and ileal digesta were measured after ashing the sample and treating the ash with boiling
8
9 158 4M hydrochloric acid (Siriwan *et al.*, 1993).

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11
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13 159 Amino acids were separated using a Beckman Model 6300 autoanalyser.
14
15 160 Detection was carried out at 570 and 440 nm after postcolumn derivatisation of the
16
17 161 amino acids with ninhydrin. Three replicates of all analyses were performed. The
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19 162 concentrate samples (100 mg) were hydrolysed with 6 N hydrochloric acid at 110°C for
20
21 163 24 h in nitrogen atmosphere. The proportion of hydrochloric acid was 1 ml/mg of
22
23 164 protein in the sample (Finley, 1985). Prior to acid hydrolysis, the samples were analysed
24
25 165 by performic acid oxidation. The hydrolysate was filtered through Whatman 541 paper
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27 166 and the volume adjusted to 100 ml of solution with Milli-Q water. An amount of 2ml of
28
29 167 solution was evaporated to dryness in a rotary evaporator at 40°C. The hydrolysate
30
31 168 residue was re-dissolved in 200 µL of 0.2 M citrate buffer (pH 2.2) containing
32
33 169 norleucine as internal standard and diluted to 1000 µL with the same buffer, and 50 µL
34
35 170 was injected into the autoanalyser. Determination of the amino acid tryptophan was not
36
37 171 possible under the conditions of analysis employed.

172 **Calculations and statistical analysis**

173 Apparent ileal digestibilities (AID, %) of crude protein (CP) and amino acid (AA) were
174 calculated using the following formula: $100 - [100 \times (\text{AIA concentration in feed} \div \text{AIA}$
175 $\text{concentration in ileal digesta}) \times (\text{CP or AA concentrations in ileal digesta} \div \text{CP or AA}$
176 $\text{concentrations in feed})]$.

177 In experiment 1, data were analysed as a 2 x 2 factorial design with two
178 concentrations of AP from 1 d of age to 3 weeks (3.5 and 2.2 g/kg) and for 3 to 6 weeks
179 (2.7 and 1.4 g/kg), and two levels of phytase (0 and 500 FTU/kg) in each period.

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3 180 Treatment 1 was considered as a positive control, adequate in AP and Ca; therefore,
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5 181 phytase was not added. This control treatment was excluded of statistical analysis. The
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8 182 statistical model used was: $Y_{ijk} = \mu + P_i + E_j + PE_{ij} + R_k + e_{ijk}$, where Y_{ijk} is the individual
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10
11 183 observation, μ is the experimental mean, P_i is the available phosphorus effect, E_j is the
12
13 184 enzyme phytase effect, R_k is the replication effect, and e_{ijk} is the error term.

15 In experiment 2, data were analysed as a 3 x 2 factorial design with two levels of
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17
18 186 phytase (0 and 750 FTU/kg diet) and three concentrations of citric acid (0, 20 and 50
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20
21 187 g/kg). The statistical model used was: $Y_{ijk} = \mu + E_i + C_j + EC_{ij} + R_k + e_{ijk}$, where Y_{ijk} is
22
23 188 the individual observation, μ is the experimental mean, E_i is the enzyme phytase effect,
24
25 189 C_j is the citric acid effect, R_k is the replication effect, and e_{ijk} is the error term. In both
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28 190 experiments, data were subjected to analysis of variance using the General Linear
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30 191 Models (GLM) procedures of SAS[®] (SAS Institute, 2001). Significant differences
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32 192 among treatment means were determined at $P < 0.05$ by Duncan's multiple range test.
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35 193 Comparisons between the diets with and without added phytase at each citric acid
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37 194 content were made using nonorthogonal contrast.

39 RESULTS

41 Experiment 1

44 *Weight gain, feed consumption and gain:feed*

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47 198 The effects of AP concentrations and phytase supplementation on growth performance
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49 199 have been previously published by Viveros *et al.* (2002), indicating that the decrease in
50
51 200 AP content in the diet depressed weight gain, feed consumption and gain:feed. Phytase
52
53 201 had a favourable effect on weight gain at 3 and 6 weeks of age and on feed consumption
54
55
56 202 only at 3 weeks. Gain:feed was not affected at any stage by addition of phytase.

58 203 *AID of CP and dispensable amino acids*

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3 204 The influence of AP and microbial phytase on the AID coefficients of CP and
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5 205 indispensable (essential) amino acids (EAA) of male chickens are shown in Table 3.
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7
8 206 The main effects data indicated that the decrease in AP content in the diet did not affect
9
10 207 the AID of CP and EAA of the diets. Likewise, the addition of phytase resulted in a
11
12 208 significant increase ($P<0.05$) in AID of CP (6.2 %) and all of the EAA (Arg 3.0 %; His
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14 209 4.8 %; Iso 5.2 %; Leu 4.4 %; Lys 4.7; Met 2.7 %; Phe 4.5 %; Thr 6.4 % and Val 5.3 %).
15
16
17 210 Except for crude protein, a significant interaction ($P<0.001$) among AP concentrations
18
19 211 and phytase was observed, indicating that phytase improved the amino acid
20
21 212 digestibilities only at low AP levels. Table 3 near here

24 213 *AID of dispensable amino acids*

26
27 214 The influence of AP and microbial phytase on the AID coefficients of dispensable (non
28
29 215 essential) amino acids (NEAA) of male chickens is summarised in Table 4. The main
30
31 216 effects data indicated that the decrease in AP content in the diet did not affect the AID
32
33 217 of NEAA of the diets, except for Ala, which was reduced (7.6 %; $P<0.01$). Phytase
34
35 218 addition increased ($P<0.05$ to 0.001) all of the NEAA (Asp 7.3%; Glu 3.3 %; Gly 5.8
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37 219 %; Pro 4.5 %; Ser 7.6 %; Tyr 8.6 %, and Cys 6.9 %), except Ala, which was not
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39 220 affected. Except for Ala, a significant interaction ($P<0.05$ to 0.001) among AP
40
41 221 concentration and phytase was observed, indicating that phytase improved the amino
42
43 222 acid digestibilities only at low AP levels. Table 4 near here

48 223 **Experiment 2**

50 224 *Weight gain, feed consumption and gain:feed*

52
53 225 *Main effects.* The effects of citric acid (CA) and phytase supplementation on growth
54
55 226 performance are summarised in Table 5. The main effects data indicated that the
56
57 227 increase in CA content reduced weight gain (up to 5%; $P<0.01$). However, the addition
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3 228 of CA did not significantly modify feed consumption and gain:feed. The performance of
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5 229 birds was not affected by phytase addition.

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8 230 *Contrast.* The addition of phytase to each citric acid level did not influence weight gain,
9
10 231 feed consumption and gain:feed. Table 5 near here

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12 232 *AID of CP and dispensable amino acids*

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15 233 *Main effects.* The influence of CA and microbial phytase on the AID coefficients of CP
16
17 234 and EAA of male chickens are shown in Table 6. The main effects data indicated that
18
19 235 the increase in CA content in the diet did not affect the AID of CP and EAA of the
20
21 236 diets, except Leu which was reduced (by up to 1.4%; $P<0.05$). The addition of phytase
22
23 237 increased the AID of CP (2.8%; $P<0.001$) and Met (1 %; $P<0.05$). All two-way
24
25 238 interactions were non-significant except in the case of Phe ($P<0.01$) and Thr ($P<0.05$),
26
27 239 which indicates that the effect of phytase on digestibilities of these amino acids was
28
29 240 dependent on CA content.

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32
33 241 *Contrast.* When birds were given diets without CA, adding phytase diets increased
34
35 242 crude protein ($P<0.001$), Met ($P<0.05$), Phe ($P<0.01$), and Thr ($P<0.05$) digestibilities.
36
37 243 The addition of phytase to the 50 g/kg CA diets increased crude protein ($P<0.05$)
38
39 244 digestibility. Tables 6 and 7 near here

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43 245 *AID of dispensable amino acids*

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46 246 *Main effects.* The influence of CA and microbial phytase on the AID coefficients of
47
48 247 NEAA of male chickens is shown in Table 7. The main effects data indicated that the
49
50 248 increase in CA content in the diet improved the AID of Asp (up to 1.9; $P<0.05$) and Gly
51
52 249 (up to 2.1 %; $P<0.05$) and decreased Pro (up to 5.8; $P<0.001$). The AID of NEAA was
53
54 250 not affected by phytase addition. The two-way interaction of CA and phytase on Asp
55
56 251 ($P<0.01$), Glu ($P<0.01$) Gly ($P<0.05$), Ser ($P<0.05$) and Tyr ($P<0.01$) digestibilities was
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252 also observed, which indicates that the effect of phytase on digestibilities of these amino
253 acids was dependent on CA content.

254 *Contrast.* When birds were given diets without CA, adding phytase diets increased Asp
255 ($P<0.01$), Gly ($P <0.05$), and Ser ($P<0.05$) digestibilities. The addition of phytase to the
256 20 g/kg CA diets decreased Asp ($P<0.05$), Glu ($P<0.01$), and Tyr ($P<0.05$)
257 digestibilities. Amino acid digestibilities were not influenced by phytase at the 50 g/kg
258 CA level.

259 DISCUSSION

260 The present study shows the effects of citric acid and microbial phytase
261 supplementation on performance and AID of CP, EAA, and NEAA in male chickens
262 fed a corn-soybean meal diet to 6 (Experiment 1) and 3 weeks. (Experiment 2) of age.
263 The protein/amino acid effect of microbial phytase is of considerable practical
264 significance and needs to be quantified to enable its inclusion in least-cost diet
265 formulations. The efficacy of phytase in de-phosphorylating phytate in plant-derived
266 ingredients and thereby improving its availability for pigs and poultry is established.
267 However, the same cannot be said of protein and amino acid utilisation responses to
268 microbial phytase due to a number of conflicting reports.

269 In the first experiment, results already published (Viveros *et al.*, 2002)
270 demonstrated that the phytase supplementation improved the performance of the birds
271 only in low-AP diets (1.4 g/kg), but not at higher AP concentration (2.7 g/kg). The
272 second experiment confirms these results showing that microbial phytase
273 supplementation failed to improve the performance of the chicks with similar AP levels
274 (2.5 g/kg). These results were in agreement with those reported by Qian *et al.* (1996),
275 which indicated also that microbial phytase seems to be more efficient in diets with
276 little or no inorganic P supplementation. Um and Paik (1999) in laying hens, Ravindran

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3 277 *et al.* (2000) in chickens and Keshavarz (2000) in pullets also indicated that the birds
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5 278 have a greater ability to retain P from diets with lower rather than higher non-phytate
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7 279 phosphorus (nPP) content, which may influence performance.
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10
11 280 Moreover, a decreased phytate-P digestibility in response to increasing the Ca:tP
12
13 281 ratio has been reported in rats (Nelson and Kirby, 1979), poultry (Qian *et al.*, 1996) and
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15 282 pigs (Lei *et al.*, 1994). In fact, Qian *et al.* (1997) showed that the increase observed in P
16
17 283 retention with phytase addition was negatively influenced by increasing the dietary
18
19 284 Ca:total P ratio. The extra Ca may directly repress phytase activity by competing for the
20
21 285 active sites of the enzymes (Pointillart *et al.*, 1985). Therefore, the Ca:tP ratio of 2.2:1
22
23 286 used in our experiment may also explain the lack of effect of phytase on performance.
24
25 287 However, Boling-Frankenbach *et al.* (2001) demonstrated that phytase was not affected
26
27 288 for dietary Ca and non phytate P concentrations.
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30
31 289 In experiment 2, the addition of the highest level of citric acid (50 g/kg) to low-
32
33 290 AP diets depressed weight gain. These results are similar to those published by Brenes
34
35 291 *et al.* (2003). The reason for a negative response or absence of response to citric acid is
36
37 292 unclear. Boling-Frankenbach *et al.* (2001) found a negative effect on performance of
38
39 293 chickens when adding 60 g/kg citric acid to diets containing adequate available
40
41 294 phosphorus (4.5 g/kg). Boling *et al.* (2000) and Boling-Frankenbach *et al.* (2001)
42
43 295 indicated that citric acid (20 to 60 g/kg) had a positive effect on performance only in
44
45 296 low-AP diets (1.0 to 2.5 g/kg) and with a Ca:AP ratio similar to or greater than 4:1.
46
47 297 Probably, as demonstrated by these authors, citrate addition in diets AP deficient and
48
49 298 with low Ca:AP ratio caused an exacerbation of Ca deficiency by the release of
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51 299 additional available P. Therefore, the Ca:AP ratio of 3.2:1 used in our experiment may
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53 300 support this hypothesis.
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3 301 In the first experiment, the addition of phytase (500 U/kg) increases the AID of
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5 302 CP and all of the EAA and NEAA only at low AP concentration (1.4 g/kg). These
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7 303 results are in general agreement with the findings of several studies which demonstrated
8
9 304 positive effects of supplemental phytase on ileal digestibility of nitrogen, protein or
10
11 305 amino acids in male (Ravindran *et al.*, 1999, 2000, 2001; Rutherford *et al.*, 2002), and
12
13 306 female chickens (Sebastian *et al.*, 1997), laying hens (van der Klis and Versteegh,
14
15 307 1991), and turkeys (Yi *et al.*, 1996). The effectiveness of phytase was negatively related
16
17 308 to the amount of inorganic phosphorus in the diet. Additional inorganic P and/or Ca
18
19 309 compromised the capacity of exogenous phytase to enhance AA digestibility,
20
21 310 presumably by reducing the extent of phytate hydrolysis (Ravindran *et al.*, 2000). Lei
22
23 311 and Stahl (2000) also argued that exogenous phytase is more efficacious in diets
24
25 312 containing low levels of inorganic P because the catalytic activity of phytase is strongly
26
27 313 inhibited by P, the hydrolytic end product of the reaction. Improvements in amino acid
28
29 314 digestibility support the idea that phytate and protein can form binary complexes
30
31 315 through electrostatic links of its charged phosphate groups with either the amino group
32
33 316 on arginine and lysine residues present within protein or with the terminal amino group
34
35 317 on proteins (Maenz, 2001). These complexes may be formed *de novo* at acidic pH in the
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37 318 gut from the protein bodies of oilseeds and in the protein-rich aleurone layers of cereal
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39 319 grains (Selle *et al.*, 2000). The complexing of phytate with proteins can change the
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41 320 protein structure, which in turn decreases solubility, digestibility, and functionality of
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43 321 proteins (Yi *et al.*, 1996). Another possible mechanism reported by Cowieson *et al.*
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45 322 (2004) is that phytate may interact with the gastrointestinal tract increasing N
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47 323 endogenous loss, mainly in the amino acids cystine, threonine, serine, and methionine
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49 324 (Selle *et al.*, 2000).
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3 325 In contrast, there are other studies in which dietary supplementation with
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6 326 phytase had no effect on apparent ileal digestibility of amino acids in male (Sebastian *et*
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8 327 *al.*, 1997; Onyango *et al.*, 2005; Martinez-Amezcuca *et al.*, 2006) and female chickens
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10 328 (Peter and Baker, 2001; Snow *et al.*, 2003) and turkeys (Ledoux *et al.*, 1999) or true
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12 329 amino acid digestibility in soybean meal intubated into caecectomised roosters (Biehl
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14
15 330 and Baker, 1997). In fact, in the second experiment the addition of phytase (750
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17 331 FTU/kg) did not modify the AID of EAA and NEAA, except in the case of Met.
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19 332 Likewise, Peter *et al.* (2000) and Boling-Frankenbach *et al.* (2001) did not improve
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21 333 protein or amino acid utilisation by phytase addition in several feed ingredients in
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23 334 young chicks. Probably the lack of response of phytase in the second experiment may
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25 335 have been related to dietary AP content (2.5 g/kg), as in the first experiment (2.7 g/kg),
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27 336 or to the age of the birds (3 weeks). It is generally agreed that the ability of poultry to
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29 337 utilise phytate phosphorus increases with age (Edwards *et al.*, 1989). In balance
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31 338 experiments investigating the hydrolysis of phytate by 4 and 9 week old broilers,
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33 339 Nelson *et al.* (1976) observed a slight increase in phosphorus utilisation by the older
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35 340 birds.

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41 341 Another objective of the current experiment was to evaluate the efficacy of citric
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43 342 acid (CA) in improving the digestibility of CP and AA and to observe whether phytase
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45 343 and CA may have some additive or synergistic effects. Since interactions between
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47 344 phytate and protein are mediated by cations (Gifford and Clydesdale, 1990), the
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49 345 addition of an organic acid, such as citric acid, may reduce the formation of this
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51 346 complex by chelating free cations. Given that microbial phytase is most active at pH 2.5
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53 347 and 5.5 (Simons *et al.*, 1990), and that some intestinal sections have different pH values
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55 348 (Dänicke *et al.*, 1999), the effectiveness of microbial phytase may be enhanced, at least
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57 349 in theory, by feeding it in combination with an organic acid. In fact, Maenz *et al.* (1999)

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3 350 observed that competitive chelation by compounds such as EDTA, citric acid or
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5 351 phthalic acid has the potential to decrease enzyme-resistant forms of phytic acid and
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8 352 thereby improve the efficacy of microbial phytase in hydrolysing phytic acid.
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10 353 In general, in the second experiment, the main effect data indicated that
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12 354 increasing the CA content of the diet did not affect the AID of CP, EAA and NEAA of
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15 355 the diets. Moreover, we did not observe a synergistic effect of microbial phytase and
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17 356 dietary acidification on amino acid digestibilities. The failure of citric acid to
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19 357 significantly improve digestibility of amino acids in chicks given phytase was
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21
22 358 unexpected. The reason for the absence of response is unknown. A possible explanation
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24 359 may be that the citric acid complexed with Ca and decreased its binding to phytate,
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26 360 increasing the susceptibility of the phytate to hydrolysis by enzyme. However, the
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28 361 additional P liberated could cause a decrease of the effectiveness of phytase as have
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30 362 already demonstrated Ballam *et al.* (1984), Yi *et al.* (1996) and Ravindran *et al.* (2000).
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32 363 In fact, in the second experiment, the AID of CP and the greatest part of AA were
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34 364 significantly increased in absence of CA, due probably to a lower concentration of P in
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36 365 the gut. In any case, there are no references in chicks on this subject. Similarly,
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38 366 Radcliffe *et al.* (1998) and Li *et al.* (1998) in pigs reported no synergistic effects with
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40 367 the combination of phytase and citric acid on performance and mineral digestibility.
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45 368 In conclusion, apart from the positive effect on the digestibility of phosphorus
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47 369 and some other minerals, addition of phytase to maize-soybean diets resulted in a
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49 370 positive effect on the AIA of CP and AA only in low AP diets. Likewise, the AID of CP
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51 371 and dispensable and indispensable AA were unaffected by CA addition. In our
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53 372 experimental conditions, the combination of CA and phytase did not appear to be a
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55 373 practical solution to improving amino acid digestibilities. Further research is needed to
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3 374 study interactions between phytate, minerals, protein, phytase and citric acid, and the
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5 375 effects of these interactions on nutrient digestibilities.
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Table 1. Composition of experimental diets (g/kg, as fed basis)

Ingredients	Experiment 1 ^a						Experiment 2		
	Starter (0 to 3 wk)			Grower-Finisher (3 to 6 wk)			Starter (0 to 3 wk)		
	Control	T ₂ -T ₄	T ₃ -T ₅	Control	T ₂ -T ₄	T ₃ -T ₅	T ₁ -T ₂	T ₃ -T ₄	T ₅ -T ₆
Maize	522.1	523.5	526.8	610.0	611.7	613.6	516.2	484.1	435.6
Soybean meal	374.3	375.2	376.4	291.1	291.8	292.7	385.0	392.9	405.0
Sunflower oil	45.1	45.1	45.1	45.1	45.1	45.3	58.3	62.5	69.0
Calcium carbonate	11.2	14.7	19.4	10.5	14.1	18.6	13.3	13.3	13.3
Dicalcium phosphate	19.4	13.6	5.8	15.9	9.9	2.4	7.6	7.6	7.5
Salt	2.9	2.9	1.5	2.9	2.9	2.9	3.0	3.0	3.0
DL-methionine	1.5	1.5	1.5	1.0	1.0	1.0	1.6	1.6	1.6
Trace minerals mixture ^b	3.5	3.5	3.5	3.5	3.5	3.5	4.0	4.0	4.0
Vitamins mixture ^c	10.0	10.0	10.0	10.0	10.0	10.0	1.0	1.0	1.0
Celite ^d	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Citric acid	-	-	-	-	-	-	-	20.0	50.0
Natuphos [®] 5000 ^e	-	-/+	-/+	-	-/+	-/+	-/+	-/+	-/+
Nutrient composition									
Calculated (g/kg)									
ME, MJ/kg ^f	12.6	12.6	12.6	12.7	12.7	12.7	13.0	13.0	13.0
Crude protein (N x 6.25)	223.0	223.0	223.0	190.0	190.0	190.0	230.0	230.0	230.0
Lysine	12.5	12.5	12.5	10.4	10.4	10.4	12.8	12.9	13.1
Methionine + cystine	8.8	8.8	8.8	7.6	7.6	7.6	9.0	9.0	9.0
Calcium (Ca)	9.9	9.9	9.9	8.7	8.7	8.7	8.0	8.0	8.0
Total phosphorus (tP)	7.1	6.1	4.9	6.2	5.2	4.0	6.1	6.0	6.0
Available phosphorus	4.5	3.5	2.2	3.7	2.7	1.4	2.5	2.5	2.5
Ca:tP ratio	1.39:1	1.62:1	2.02:1	1.40:1	1.67:1	2.2:1	1.31:1	1.33:1	1.33:1
Analysed (g/kg)									
Crude protein (N x 6.25)	221.0	220.8	221.9	186.6	188.8	186.7	231.0	229.0	230.0
Calcium	12.9	11.9	11.9	11.4	10.4	11.2	8.3	8.1	8.4
Phosphorus, total	8.1	7.1	5.8	7.6	6.4	5.2	6.3	6.0	6.6

^a In experiment 1, birds in the control group (T₁) were given 4.5 g/kg available P (AP) from 1 d of age to 3 wk (starter diets) and 3.7 g/kg AP from 3 to 6 wk (grower-finisher diets) without enzyme added. The AP level was reduced by 1 g/kg in each period in T₂ and T₄ and by 2.3 g/kg in each period in T₃ and T₅.

^b Mineral mix supplied the following per kg of diet: Mn, 55 mg; Zn, 50 mg; Fe, 80 mg; Cu, 5 mg; Se, 0.1 mg; I, 0.18 mg.

^c Vitamin mix supplied the following per kilogram of diet: retinol, 2.5 mg; cholecalciferol, 25 µg; α-tocopherol acetate, 7.34 mg; menadione, 1.1 mg; cyanocobalamin, 11.5 µg; riboflavin, 5.5 mg; Ca pantothenate, 11 mg; niacin, 53.3 mg; choline chloride, 1,020 mg; folic acid, 0.75 mg; biotin, 0.25 mg; delaquein, 125 mg; DL-methionine, 500 mg.

^d Celite Corp., Lompoc, CA 93436.

^e Natuphos[®] 5000 (BASF Corp., Mt. Olive, NJ 07828-1234) was used as the source of microbial phytase to provide 500 (T₄ and T₅, experiment 1) and 750 (T₂, T₄ and T₆; experiment 2) units phytase /kg diet.

^f Metabolisable energy (ME) was estimated using FEDNA Tables (2003).

Table 2. *Analysed amino acid composition of the diets (g/kg dry matter)*

	Experiment 1 (3 to 6 weeks)			Experiment 2 (1 d to 3 weeks)		
	Available phosphorus (g/kg)			Citric acid (g/kg)		
	3.7	2.7	1.4	0	20	50
Indispensable						
Arginine	13.6	13.6	13.3	14.6	16.1	16.0
Histidine	5.4	5.5	5.3	5.8	6.3	6.2
Isoleucine	7.8	7.7	7.5	8.8	9.0	9.1
Leucine	17.4	17.5	16.9	20.2	19.6	19.4
Lysine	10.8	10.8	10.1	12.1	13.0	13.0
Methionine	3.6	3.6	3.6	3.9	4.5	4.3
Phenylalanine	10.2	10.1	9.8	12.2	12.1	12.1
Threonine	6.9	7.7	7.4	8.0	8.9	8.9
Valine	8.5	8.3	8.1	8.8	9.3	9.2
Dispensable						
Alanine	12.5	12.0	10.7	10.5	10.7	10.7
Aspartate	17.4	19.9	19.1	22.1	24.5	24.8
Glutamate	30.0	32.1	28.0	39.4	41.7	41.8
Glycine	8.7	8.6	8.5	8.4	9.4	9.4
Proline	10.6	10.5	10.1	12.6	11.9	11.7
Serine	9.5	10.3	10.0	11.5	11.9	12.0
Tyrosine	7.3	7.6	7.4	8.0	8.5	8.2
Cystine	2.3	2.3	2.3	2.4	2.7	2.6

Table 3. Effect of available phosphorus (AP) level with and without microbial phytase on apparent ileal digestibility coefficient of protein and indispensable amino acids in 42-d-old male chickens¹. Experiment 1.

Treatment	AP (g/kg)	Phytase (U/Kg)	Digestibility coefficients									
			CP	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val
1 (Control)	3.7	-	0.794 ^a	0.834 ^b	0.791 ^c	0.774 ^{bc}	0.790 ^c	0.787 ^{bc}	0.823 ^c	0.779 ^{bc}	0.686 ^b	0.763 ^{bc}
2	2.7	-	0.784 ^{ab}	0.878 ^a	0.835 ^{ab}	0.818 ^{ab}	0.838 ^{ab}	0.836 ^{ab}	0.880 ^{ab}	0.821 ^{ab}	0.748 ^a	0.804 ^{ab}
3	1.4	-	0.749 ^b	0.836 ^b	0.776 ^c	0.754 ^c	0.777 ^c	0.755 ^c	0.838 ^{bc}	0.764 ^c	0.683 ^b	0.741 ^c
4	2.7	500	0.806 ^a	0.864 ^{ab}	0.817 ^{bc}	0.799 ^{bc}	0.817 ^{bc}	0.808 ^{abc}	0.858 ^{bc}	0.797 ^{bc}	0.729 ^b	0.784 ^{bc}
5	1.4	500	822 ^a	0.900 ^a	0.867 ^a	0.850 ^a	0.865 ^a	0.855 ^a	0.902 ^a	0.854 ^a	0.789 ^a	0.839 ^a
Pooled SEM			0.034	0.031	0.033	0.039	0.036	0.045	0.033	0.038	0.047	0.039
Main effects ²												
AP												
2.7			0.795	0.872	0.827	0.809	0.828	0.823	0.870	0.810	0.740	0.795
1.4			0.786	0.868	0.821	0.802	0.821	0.805	0.870	0.809	0.736	0.790
Phytase												
0			0.767	0.857	0.806	0.786	0.807	0.796	0.859	0.792	0.716	0.773
500			0.814	0.883	0.844	0.827	0.843	0.833	0.882	0.828	0.762	0.814
Statistical significance			-----					Probabilities				
AP effect			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Phytase effect			**	*	**	*	*	*	*	*	*	*
AP x phytase			NS	***	***	***	***	***	***	***	**	***

^{a-c} Means in columns with no common superscript differ significantly ($P < 0.05$).

¹ Values are means of 6 replications of 8 chicks each.

² Data analysed as a 2 x 2 factorial design, excluding the control group.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS not significant.

Table 4. Effect of available phosphorus (AP) level with and without microbial phytase on apparent ileal digestibility coefficient of dispensable amino acids in 42-d-old male chickens¹. Experiment 1.

Treatment	AP (g/kg)	Phytase (U/Kg)	Digestibility coefficients							
			Ala	Asp	Glu	Gly	Prol	Ser	Tyr	Cys
1 (Control)	3.7	-	0.806 ^{ab}	0.739 ^c	0.808 ^c	0.745 ^{bc}	0.788 ^{bc}	0.747 ^{bc}	0.674 ^{ab}	0.713 ^{bc}
2	2.7	-	0.841 ^a	0.769 ^{bc}	0.870 ^{ab}	0.779 ^{ab}	0.827 ^{ab}	0.802 ^{ab}	0.713 ^{ab}	0.745 ^{ab}
3	1.4	-	0.750 ^b	0.757 ^{bc}	0.838 ^{bc}	0.711 ^c	0.774 ^c	0.705 ^c	0.650 ^b	0.670 ^c
4	2.7	500	0.811 ^{ab}	0.787 ^b	0.861 ^{ab}	0.751 ^{bc}	0.809 ^{bc}	0.779 ^{ab}	0.723 ^{ab}	0.714 ^{bc}
5	1.4	500	0.779 ^{ab}	0.845 ^a	0.900 ^a	0.820 ^a	0.861 ^a	0.837 ^a	0.755 ^a	0.791 ^a
Pooled SEM			0.049	0.035	0.035	0.038	0.030	0.052	0.067	0.038
Main effects ²										
AP										
2.7			0.827	0.778	0.866	0.766	0.819	0.792	0.718	0.731
1.4			0.764	0.801	0.869	0.765	0.817	0.771	0.703	0.730
Phytase										
0			0.795	0.763	0.854	0.745	0.801	0.753	0.682	0.707
500			0.794	0.819	0.883	0.788	0.837	0.811	0.740	0.756
Statistical significance			Probabilities				Probabilities			
AP effect			**	NS	NS	NS	NS	NS	NS	NS
Phytase effect			NS	***	*	*	*	*	*	*
AP x phytase			NS	*	*	***	***	**	*	***

^{a-c} Means in columns with no common superscript differ significantly ($P < 0.05$).

¹ Values are means of 6 replications of 8 chicks each.

² Data analysed as a 2 x 2 factorial design, excluding the control group.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS not significant.

Table 5. Effect of citric acid (CA) and phytase on weight gain, feed consumption, and gain:feed in 21-d-old male chickens^a. Experiment 2

Treatment	CA (g/kg)	Phytase ³ (U/Kg)	Weight gain (g)	Feed intake (g)	Gain:feed (g/g)
1	0	0	583 ^a	782	1.36
2	0	750	565 ^{abc}	778	1.38
3	20	0	567 ^{ab}	775	1.37
4	20	750	557 ^{abc}	750	1.33
5	50	0	533 ^c	729	1.37
6	50	750	540 ^{bc}	756	1.37
Pooled SEM			67.95	50.35	0.04
Main effects ²					
CA					
0			574 ^a	780	1.37
20			562 ^a	763	1.35
50			537 ^b	742	1.37
Phytase					
0			561	762	1.36
750			554	761	1.36
Statistical significance					
CA effect			**	NS	NS
Phytase effect			NS	NS	NS
CA x phytase			NS	NS	NS
Contrast Diet 1 vs 2			NS	NS	NS
Contrast Diet 3 vs 4			NS	NS	NS
Contrast Diet 5 vs 6			NS	NS	NS

^{a-c} Means in columns with no common superscript differ significantly ($P < 0.05$).

¹ Values are means of 6 replications of 8 chicks each.

² Data analysed as a 3 x 2 factorial design.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS not significant.

Table 6. Effect of citric acid (CA) and phytase on apparent ileal digestibility coefficient of protein and indispensable amino acids in 21-d-old male chickens¹. Experiment 2

Treatment	CA (g/kg)	Phytase (U/Kg)	Digestibility coefficients									
			CP	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val
1	0	0	0.855 ^c	0.918 ^b	0.877 ^b	0.889	0.911 ^{ab}	0.916	0.925 ^b	0.887 ^b	0.899 ^b	0.884
2	0	750	0.885 ^a	0.936 ^a	0.910 ^a	0.907	0.921 ^a	0.932	0.948 ^a	0.912 ^a	0.856 ^{ab}	0.894
3	20	0	0.862 ^{bc}	0.942 ^a	0.919 ^a	0.904	0.912 ^{ab}	0.931	0.942 ^a	0.903 ^{ab}	0.868 ^a	0.895
4	20	750	0.881 ^a	0.931 ^{ab}	0.905 ^{ab}	0.890	0.902 ^{ab}	0.916	0.943 ^a	0.887 ^b	0.844 ^{ab}	0.876
5	50	0	0.856 ^c	0.933 ^{ab}	0.900 ^{ab}	0.894	0.897 ^b	0.918	0.932 ^{ab}	0.884 ^b	0.837 ^b	0.876
6	50	750	0.876 ^{ab}	0.937 ^a	0.905 ^{ab}	0.891	0.902 ^{ab}	0.920	0.936 ^{ab}	0.891 ^b	0.845 ^{ab}	0.873
Pooled SEM			0.012	0.014	2.37	0.015	0.016	0.014	0.012	0.015	0.020	0.024
Main effects ²												
CA												
	0		0.870	0.927	0.893	0.899	0.916 ^a	0.924	0.938	0.899	0.842	0.889
	20		0.873	0.936	0.911	0.896	0.906 ^{ab}	0.923	0.943	0.895	0.855	0.885
	50		0.866	0.935	0.903	0.892	0.900 ^b	0.919	0.934	0.888	0.841	0.874
Phytase												
	0		0.857	0.930	0.898	0.896	0.906	0.921	0.933	0.891	0.843	0.885
	750		0.881	0.935	0.907	0.896	0.908	0.923	0.943	0.897	0.848	0.881
Statistical significance												
CA effect			NS	NS	NS	NS	*	NS	NS	NS	NS	NS
Phytase effect			***	NS	NS	NS	NS	NS	*	NS	NS	NS
CA x phytase			NS	NS	NS	NS	NS	NS	NS	**	*	NS
Contrast Diet 1 vs 2			***	NS	NS	NS	NS	NS	*	**	*	NS
Contrast Diet 3 vs 4			NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Contrast Diet 5 vs 6			*	NS	NS	NS	NS	NS	NS	NS	NS	NS

^{a-c} Means in columns with no common superscript differ significantly ($P < 0.05$).

¹ Values are means of 6 replications of 8 chicks each.

² Data analysed as a 3 x 2 factorial design.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS not significant.

Table 7. Effect of citric acid (CA) and phytase on apparent ileal digestibility coefficient of dispensable amino acids in 21-d-old male chickens¹. Experiment 2

Treatment	CA (g/kg)	Phytase (U/Kg)	Digestibility coefficients							
			Ala	Asp	Glu	Gly	Prol	Ser	Tyr	Cys
1	0	0	0.871	0.872 ^c	0.932 ^{bc}	0.833 ^b	0.892 ^{ab}	0.869 ^b	0.778 ^b	0.799 ^{ab}
2	0	750	0.891	0.894 ^{ab}	0.940 ^{ab}	0.860 ^a	0.913 ^a	0.894 ^a	0.809 ^{ab}	0.822 ^{ab}
3	20	0	0.875	0.909 ^a	0.950 ^a	0.874 ^a	0.872 ^{bc}	0.888 ^{ab}	0.831 ^a	0.843 ^a
4	20	750	0.874	0.890 ^{bc}	0.931 ^{bc}	0.856 ^{ab}	0.851 ^c	0.871 ^b	0.777 ^b	0.809 ^{ab}
5	50	0	0.881	0.886 ^{bc}	0.923 ^c	0.856 ^{ab}	0.845 ^c	0.866 ^b	0.766 ^b	0.783 ^b
6	50	750	0.864	0.895 ^{ab}	0.938 ^{abc}	0.858 ^{ab}	0.857 ^c	0.875 ^{ab}	0.809 ^{ab}	0.813 ^{ab}
Pooled SEM			0.020	0.014	0.011	0.019	0.024	0.017	0.035	0.034
Main effects ²										
CA										
0			0.881	0.883 ^a	0.936	0.847 ^b	0.903 ^a	0.881	0.793	0.811
20			0.874	0.898 ^a	0.940	0.864 ^a	0.861 ^b	0.879	0.801	0.824
50			0.872	0.890 ^{ab}	0.930	0.857 ^{ab}	0.851 ^b	0.870	0.788	0.798
Phytase										
0			0.875	0.888	0.934	0.853	0.870	0.873	0.789	0.807
750			0.877	0.890	0.936	0.858	0.873	0.880	0.798	0.814
Statistical significance										
CA			NS	*	NS	*	***	NS	NS	NS
Phytase			NS	NS	NS	NS	NS	NS	NS	NS
CA x phytase			NS	**	**	*	NS	*	**	NS
Contrast Diet 1 vs 2			NS	**	NS	*	NS	*	NS	NS
Contrast Diet 3 vs 4			NS	*	**	NS	NS	NS	*	NS
Contrast Diet 5 vs 6			NS	NS	NS	NS	NS	NS	NS	NS

^{a-c} Means in columns with no common superscript differ significantly ($P < 0.05$).

¹ Values are means of 6 replications of 8 chicks each.

² Data analysed as a 3 x 2 factorial design.

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS not significant.