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In vivo and *in vitro* digestibility of plant ingredients and diets by *Bacillus* phytases in tilapia, *Oreochromis mossambicus*

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Abstract. This study aimed to evaluate four *Bacillus* phytases for their efficacy in making plant-based diets bioavailable to tilapia (*Oreochromis mossambicus*) using *in vivo* digestibility measurement and to determine the *in vitro* level of dephosphorylation. The four *Bacillus* strains used were *B. pumilus*, *B. megaterium*, *B. coagulans*, and *B. licheniformis*. Phytase activities varied between bacterial sources as well as between feed ingredients. For the cassava leaf meal, Pi released was highest in *B. pumilus* and was not significantly different from those of *B. megaterium* and *B. licheniformis*. For the soybean meal, Pi release was in this decreasing order: *B. megaterium* > *B. pumilus* > *B. coagulans* > *B. licheniformis* phytase. For the corn meal, addition of *B. licheniformis* phytase to the reaction mixture resulted in significantly the highest Pi released followed by *B. coagulans* phytase which was not significantly different from that of *B. megaterium* phytase which released the lowest Pi. Pi released by *B. pumilus* phytase from corn meal was not significantly different from the lowest Pi release of *B. megaterium* phytase. The apparent digestibility coefficient (ADC) values for the feed dry matter (DM) ranged from 86.3 to 88.3% and were not significantly different from each other ($p > 0.05$).
Key Words: phytases, *Bacillus* sp., *in vivo* digestibility, *in vitro* dephosphorylation, tilapia.

Introduction. In this paper, we have shown that the three phytases from *Bacillus megaterium*, *B. licheniformis* and *B. pumilus* did not enhance growth performance but mineralization of bones and scales were improved (Dechavez & Serrano 2012). The most remarkable finding was the marked improvement of P retention and lessening of fecal P and P load in all the *Bacillus* phytase-supplemented diets indicating that ameliorating water quality was the main benefit of incorporating these bacterial phytases into the diet of the tilapia *Oreochromis mossambicus*.

The major storage form of P in cereals, pollen, legumes and oilseeds is phytic acid or phytate (myo-inositol hexakis dihydrogen phosphate). Phytate influences the functional and nutritional properties of these plant feed ingredients by forming complexes with proteins and minerals thereby decreasing their bioavailability. Phytate occurs primarily as salts of mono- and divalent cations, e.g. Ca-Mg-K salt in soybean (Reddy et al 1989). Under low acidic pH, an ionic binding occurs between the basic P groups of phytate and protonized amino acid (lysil, histidyl and arginyl) residues (De Rham & Jost 1979; Fretzdorff et al 1995). Possible mechanisms include direct binding of phytate to protonated α-NH_2 terminal groups and ε-NH_2 groups of lysine residues and a multivalent cation-mediated interaction (Cheryan 1980). By binding to plant proteins, phytate decreases their solubility, therefore reducing their nutritive value. Phytic acid also interacts with enzymes such as trypsin, pepsin, α-amylase and β-galactosidase resulting in decreased activities (Singh & Krikorian 1982; Deshpande & Cheryan 1984; Inagawa et al 1987).

About two-thirds of the total P in plant protein meals such as soybean meal is in the form of phytate (Ravindran et al 1995) and its bioavailability is essentially nil to fish (NRC 1993). Addition of phytase to high phytate striped bass diets improves the absorption of utilization of P (Hughes & Soares 1998). Dietary phytase also improves the

nutritive value of canola protein concentrate and decreases P output in the case of rainbow trout *Oncorhynchus mykiss* (Forster et al 1999). Several authors report similar results for different species like channel catfish *Ictalurus punctatus* (Li & Robinson 1996), African catfish *Clarias gariepinus* (Van Weerd et al 1999), common carp *Cyprinus carpio* (Schafer et al 1995) and *Pangasius pangasius* (Debnath et al 2005a). Adding microbial phytase to diets in fish increase phytate hydrolysis and the availability of P and other minerals that may be chelated by phytic acid (Lei et al 1993; Adeola et al 1995). It is reportedly effective in enhancing the bioavailability of P considerably, thereby reducing the fecal P output.

There are two categories of phytase feed enzymes depending on the site where the hydrolysis of the phytate molecule is initiated: 3-phytase (EC 3.1.3.8) preferentially liberates the P moiety at position C3 and 6-phytase (EC 3.1.3.26) which commences at position C6 of the myo-inositol hexaphosphate ring (Selle & Ravindran 2007). Microorganisms such as the *Bacillus* normally produce the 3-phytases and plants the 6-phytases.

In view of this, the present study aims to evaluate four *Bacillus* phytases for their efficacy in making plant-based diets bioavailable to sex-reversed tilapia *O. mossambicus* using *in vivo* digestibility measurement and to determine the *in vitro* level of dephosphorylation. The four *Bacillus* strains used were *B. pumilus*, *B. megaterium*, *B. coagulans* and *B. licheniformis*.

Material and Method. The experiments were done from June to October 2008 at the hatchery facility of the Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas in Iloilo, Philippines.

Bacterial phytase production. Production of phytase was as described in Dechavez et al (2011). Pure isolates of *Bacillus* strains namely *B. pumilus* (acc. No. 1513), *B. coagulans* (Acc. No. 1510), *B. megaterium* (Acc. No. 1643) and *B. licheniformis* (Acc. 1035) were subcultured in phytase screening medium. Strain/s that produce clear zones on the screening medium were tested for phytase production in a medium containing 10 g L^{-1} sodium phytate as a substitute for the Pi. Positive strain/s was inoculated in LB (*Luria bertani* medium) agar plate. Single colony of the positive strain/s were re-inoculated in LB and incubated at 37^{o}C, centrifuged and the supernatant was used for the *in vitro* and *in vivo* assays.

In vitro digestibility determination. This experiment involved an *in vitro* evaluation of the release of P in cassava leaf (*Manihot esculenta*) meal, corn (*Zea mays*) meal and soybean (*Glycine max*) meal using the four *Bacillus* species. Dried and pulverized meals and the crude enzyme were allowed to react for a determined period after which free Pi was measured spectrophotometrically. The same assay was done using the standard substrate (sodium phytate) as positive control. The Pi and phytic acid content of the leaf and bean meals were evaluated before and after incubation. Briefly, 0.40 g dry meal was mixed with 20 ml 100 mM sodium acetate buffer pH 6.0 containing 2 mM $CaCl_2$, a 600 µL aliquot of this solution was mixed with 300 µL enzyme solution (*Bacillus* sp.) and incubated in a water bath under constant shaking for 1 h at 37^{o}C. The reaction was stopped by adding 900 µL 5% tetrachloroacetic acid (TCA). The reaction mixture was clarified by centrifugation and the supernatant was used for P analysis. A 0.5 ml aliquot of the supernatant was mixed with 2.5 ml MS solution (0.024 M sodium molybdate in 0.25 M sulfuric acid) and 0.25 ml Elon solution (3% sodium sulfite and 1% p–methylamino phenol sulfate), incubated at room temperature for 60 min and optical density read at 700 nm (Yin et al 2006). The amount of dissolved Pi in mg was estimated using ammonium phosphate as standard.

In vivo digestibility of dry matter

Experimental diet. Five experimental diets were formulated using plant ingredients and into which bacterial phytase (*B. pumilus*, *B. coagulans*, *B. megaterium* or *B. licheniformis*) were incorporated; the diet without phytase was used as the control diet

(Table 1). The feed was considered nutritionally adequate for the sex-reversed tilapia *O. mossambicus*. The resulting pellets after air drying were kept in the freezer (-20^{o}C) until use; the pellets were thawed a day before feeding and sprayed with the bacterial phytase solution and air dried before providing to the experimental fish. Chromic oxide was incorporated in the feed at 5g kg^{-1} dry matter as inert marker to estimate digestibility.

Table 1

Composition of plant-based test diets fed to sex reversed tilapia *O. mossambicus*

Ingredient	*Amount (g/100g diet)*
Fish meal	15.00
Soybean meal	41.09
Corn meal	34.91
Cassava leaf meal	5.00
Cassava starch (binder)	(5.00)
Cod liver oil	2.00
Vitamins/mineral mix	2.00
Bacterial phytase	500 FTU
Chromic oxide	0.50
Proximate composition	*(% dry matter)*
Moisture	4.34
Crude protein	35.24
Crude fat	1.41
Crude fiber	2.76
Ash	0.33
Nitrogen Free Extract	60.26

Experimental fish. Sex reversed tilapia *O. mossambicus* juveniles (62.41g ABW) were acclimatized in a 1 ton fiber glass tank in a seawater recirculating system and fed twice daily with the formulated plant based diets. Fish were randomly divided into 15 50-L aquaria at a density of ten (10) fish per aquarium. Each aquarium was connected to an Erlenmeyer flask that served as a feces collector. All the aquaria constituted a recirculating water system with a flow rate of 600 ml min^{-1} and sufficient aeration (Figure 1).

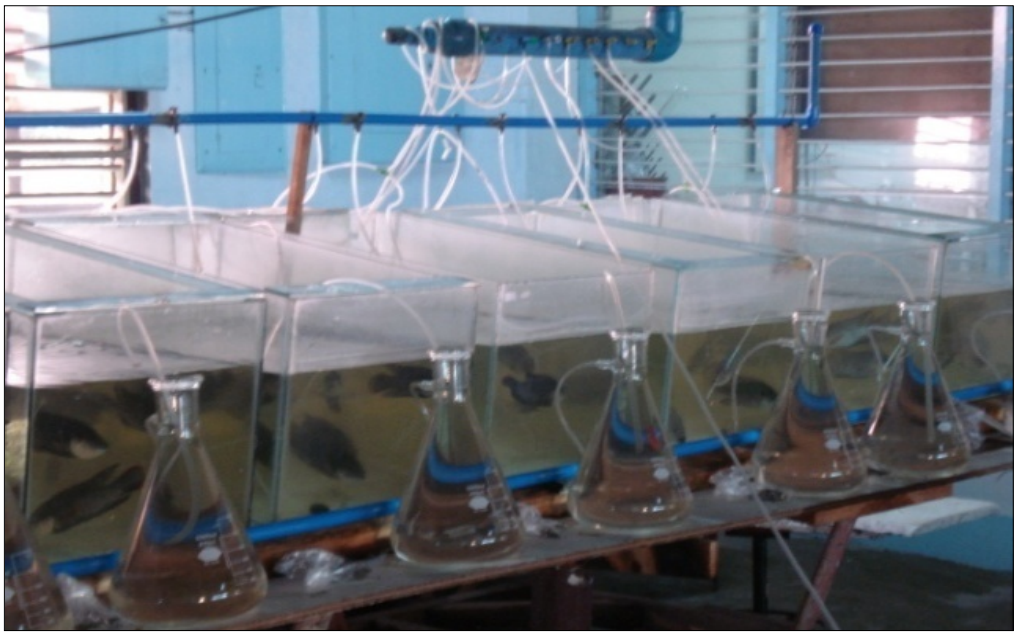

Figure 1. Digestibility set up for the measurement of in vivo apparent digestibility coefficients in juvenile *O. mossambicus*.

Digestibility trial. Experimental diets were fed to the fish twice daily at 09:00 and 16:00 h to satiation for 2 weeks. Uneaten feeds were carefully siphoned off after the last feeding in the afternoon. Following tank cleaning, collection of feces was done in the morning at 07:00 h by siphoning the feces from an improvised fecal collector. The fecal collector set up accumulated the fecal matter by a flow through system in which a silicon tube was positioned at the bottom of the tilted aquarium flowing through an Erlenmeyer flask positioned beside the aquarium; an outflow pout of the flask was positioned near the mouth of the flask in which a silicon tube was fitted enabling surface water in the flask to spill over while the fecal matter settled at the bottom of the flask by gravity. The daily collected feces were centrifuged at 2,000 rpm for 2 min and kept frozen at -20^{o}C until sufficient samples per treatment had been obtained.

Experimental diets and fecal samples were subjected to proximate analysis and to chromic oxide analysis (Furukawa & Tsukahara 1966). Pi was determined according to the method by Lovell (1975) and Pearson (1977).

The apparent digestibility coefficient (ADC) of dry matter (DM) in feed was estimated using the following formula:

$$ADC = 100 - [(\%Cr_d / \%Cr_f) \times (\%DM_f / \%DM_d) \times 100]$$

where: $\%Cr_f$ is % Cr in dried feces; $\%Cr_d$ is % Cr in dried diet; $\%DM_d$ is % nutrient in dried diet; $\%DM_f$ is % nutrient in dried feces.

Statistical analysis. Differences between treatment means on the phytase activity of the different ingredients and apparent digestibility coefficient of feeds were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's Post-hoc test for the comparison of significantly different means at alpha=0.05. Data were tested for homogeneity of variance and normality of data prior to ANOVA.

Results and Discussion

Enzymic dephosphorylation of feed ingredients. Phytase activities varied between bacterial sources as well as between feed ingredients. For the cassava leaf meal, Pi released was highest in *B. pumilus* (961 mg kg^{-1}) but was not significantly different from those of *B. megaterium* (862 mg kg^{-1}) and *B. licheniformis* (852 mg kg^{-1}). *B. coagulans* phytase released significantly the lowest Pi (491 mg kg^{-1}) (Figure 2).

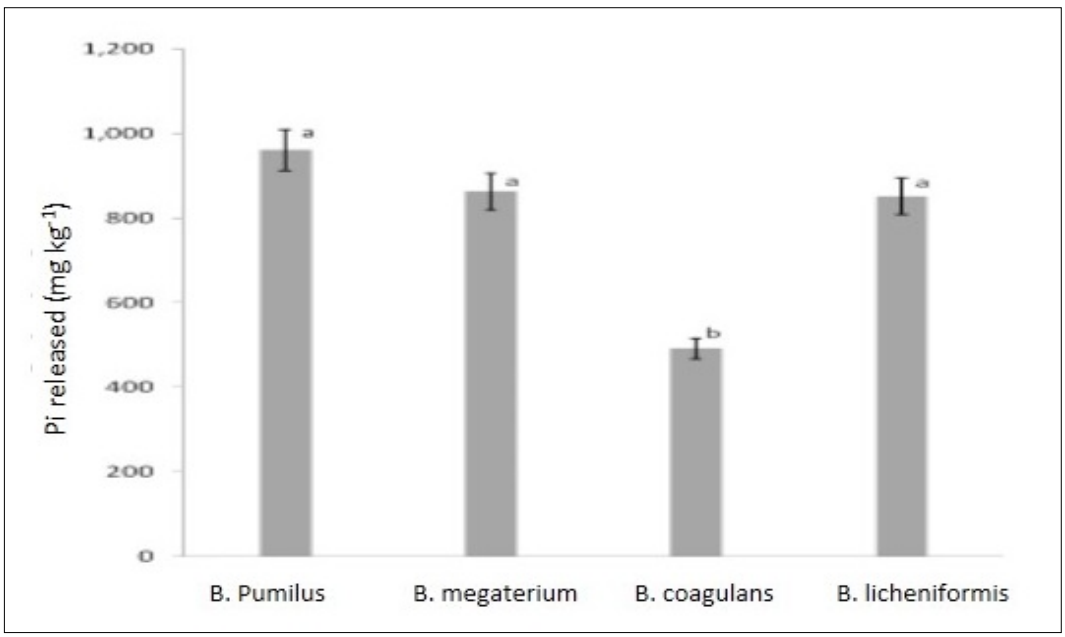

Figure 2. The P_i released from cassava leaf meal using different bacterial phytase sources after 1 h incubation at pH 6.0 at 37^{o}C (300µl enzyme + 600µl substrate + 900 µl 5% TCA). Bars represent means ± S.D. Means with different letters are significantly different ($p<0.05$).

For the soybean meal, *B. megaterium* phytase displayed significantly the highest amount of P_i released (1,212 mg Pi kg^{-1}) while the *B. licheniformis* phytase significantly the lowest (732 mg Pi kg^{-1}) (Figure 3). Phytases of *B. pumilus and B. coagulans* displayed intermediate activities that were significantly different from each other.

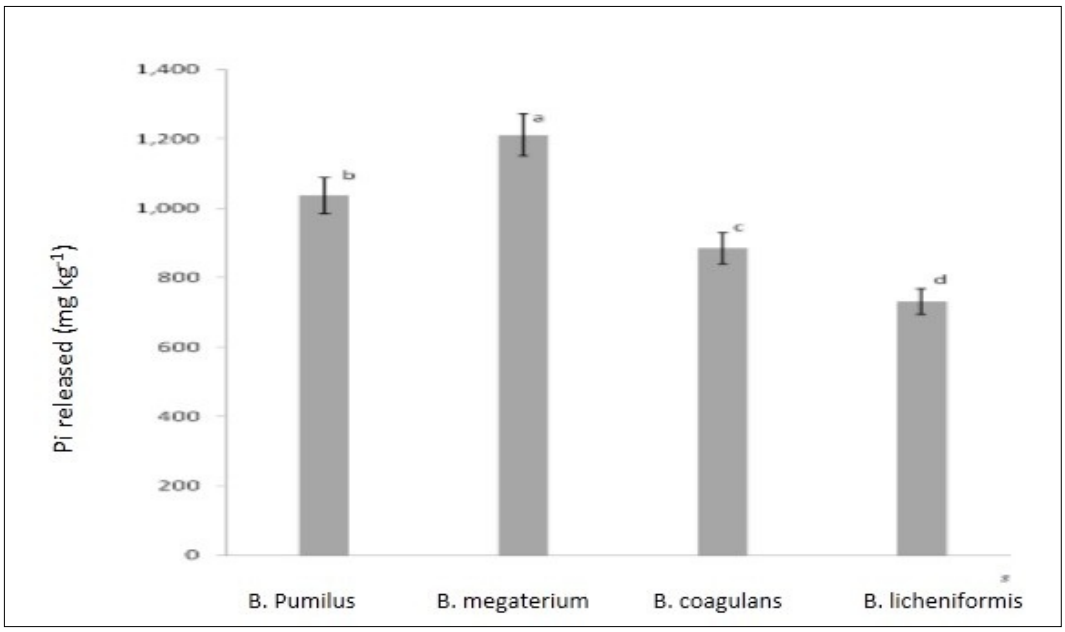

Figure 3. The Pi released from soybean meal using different bacterial phytase sources after 1 h incubation at pH 6.0 at 37°C (300µl enzyme + 600µl substrate + 900 µl 5% TCA). Bars represent means ± S.D. Means with different letters are significantly different ($p < 0.05$).

For the corn meal, *B. licheniformis* phytase resulted in significantly the highest Pi released followed by *B. coagulans* which was not significantly different from that of *B. megaterium* phytase which released the lowest Pi. Pi released by *B. pumilus* phytase was not significantly different from the lowest Pi release of *B. megaterium* phytase (Figure 4).

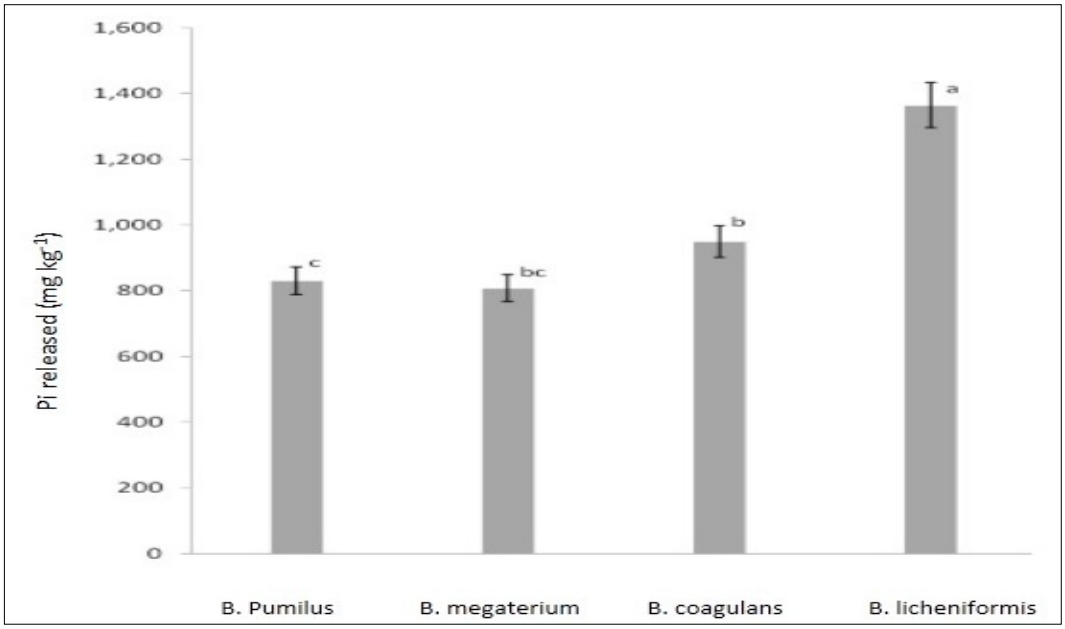

Figure 4 The Pi released from corn meal using different bacterial phytase sources after 1 h incubation at pH 6.0 at 37°C (300µl enzyme + 600µl substrate + 900 µl 5% TCA). Bars represent means ± S.D. Means with different letters are significantly different ($p < 0.05$).

Apparent digestibility. The apparent digestibility coefficient (ADC) values for the feed DM ranged from 86.3 to 88.3% and were not significantly different from each other ($p > 0.05$) (Table 2).

Table 2

Apparent digestibility coefficient of feeds (%)

Treatments	*ADC of DM (%)**
Control (without bacterial phytase)	87.4 ± 0.1
B. pumilus	86.4 ± 0.3
B. megaterium	88.3 ± 0.3
B. coagulans	86.3 ± 0.1
B. licheniformes	87.8 ± 0.2

*****ADC of DM = Apparent Digestibility Coefficient of DM of feed

In the present study the apparent digestibility coefficients (ADCs) of the plant-based diet tested i*n O. mossambicus* with or without bacterial phytase supplementation ranged from 86.3% to 88.3% and were not significantly different from each other. In the diet of African catfish *C. gariepinus*, an experiment was conducted in which phytase (Natuphos 5000) was supplemented in increasing amounts (from 13 to 1020 units kg^{-1} of diet) (Van Weerd et al 1999). Only the ADC of P was increased with increasing phytase supplementation; ADCs of dry matter, protein and energy were similar except for the treatment with 1000 units of phytase incorporated. In rainbow trout *O. mykiss*, Lanari et al (1998) fed the fish two diets containing 33% soybean meal and differed only by the addition of 1000 U phytase kg^{-1} in one diet. No differences in ADCs of dry matter, crude protein, fat, ash and gross energy except that of P. Other studies that reported no improvement in the ADC of protein were those in striped bass *Morone saxatilis* (Papatryphon et al 1999) and tilapia *O. niloticus* (Riche et al 2001). The similar ADCs of dry matter of diets supplemented with phytase from various *Bacillus* strains at 500 FTU kg^{-1} diet in the present study is in agreement with the above findings.

Reports of improved ADCs of crude protein have also been made in some fish species. In rainbow trout, Vielma et al (2004) report that protein digestibility is increased by phytase supplementation to a semipurified soybean meal-based diet. Cheng & Hardy (2002) found that phytase supplementation in expelled soybeans increased ADC of crude protein significantly compared to ADC in raw soybeans. Similar results were also found in *P. pangasius* (Debnath et al 2005b), carps (Bai et al 2004) and rainbow trout (Forster et al 1999; Sugiura et al 2001).

In poultry, *in vitro* dephosphorylation of feeds have been conducted by Zyla et al (2004) but in a more elaborate method to simulate the conditions in the gastrointestinal tract (GIT) of the chicken. They subjected the feed samples to a procedure of multiple digestions – pepsin and pancreatin digestion, preincubation at pH 5.8 in a tube, heated at $40^{o}C$ for 30 min, then 0.5 mL of 1.5 M HCl and 3,000 U of pepsin were added, then 1M $NaHCO_3$ containing 5.6 mg mL^{-1} was added dropwise with constant stirring, dialyzed, samples of the dialysate were withdrawn for Pi determination. Thus, the method in the present study was comparatively much simple in approach and did not attempt to simulate the conditions of the actual processes in the GIT of tilapia. In this light, results of *in vitro* enzymic dephosphorylation in the present study served as general tendencies for *Bacillus* phytases in hydrolyzing the phosphate at the C3 position. Future research should look at the Pi concentration in relation to the *in vivo* digestibility of dietary P.

From the results of the *in vitro* dephosphorylation, it appeared that *B. pumilus, B. megaterium and B. licheniformis* phytases were effective in dephosphorylating the cassava leaf meal. Phytate P from soybean meal and corn meal were best dephosphorylated by *B. megaterium* and *B. licheniformis,* respectively, more than did the other strains. Different ingredients have varying amounts of phytate P (Nelson 1967) and different locations of phytate P within the plant (Ravindran et al 1995). It has been demonstrated that the increase in P is primarily due to an increase in phytate P (Barrier-Guillot et al 1996).

Conclusions. The apparent digestibility coefficient (ADC) values for the feed DM ranged from 86.3 to 88.3% and were not significantly different from each other. Phytase activities varied between *Bacillus* strains as well as between feed ingredients. For the cassava leaf meal, Pi released was highest in *B. pumilus* but was not significantly different from those of *B. megaterium* and *B. licheniformis*. For the soybean and corn meals, *B. megaterium* and *B. licheniformis* phytases, respectively, displayed significantly the highest amount of Pi released

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