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# *In vitro* assessment of enzymatic phytate dephosphorylation during digestive process of different feeds and feed ingredients

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ABSTRACT

*In vivo* studies of the digestive process are long, expensive and difficult to rationalize, whereas *in vitro* systems may give more accessible insight into parts of this process. The purpose of this study was to show the ability of a three-step simulation of monogastric animals' digestive system to estimate phytate hydrolysis and how it is affected by feed composition. Several feed ingredients: wheat, maize, soybean meal and rapeseed meal and complete diets: a wheat-maize-soybean-meal-based diet, a maize-soybean-meal diet and a wheat-maize-rapeseed-meal diet were treated using an adaptation of a described *in vitro* digestion simulation system in the presence of increasing doses of phytase. A strong dependence of phytate hydrolysis on the feed ingredient used was obtained: phosphorus releases were 0.3, 0.8, 1.0 and 1.6 g/kg at 0 U/kg of phytase supplementation for maize, soybean meal, wheat and rapeseed meal respectively and 1.2, 2.9, 1.7 and 3.9 at 1000 U/kg of bacterial phytase. The efficacy of enzymatic dephosphorylation of phytate was found dependent on the ingredient, which can be partially explained by their initial content in *myo*-inositol phosphates. The *in vitro* simulation was proven a useful tool to assess enzymatic dephosphorylation of phytate under different conditions.

## 1. Introduction

Phosphorus (P) is an essential nutrient in animal nutrition. It has an important structural role in bone mineralization, as a component of the crystalline structure of the skeleton, as well as in the form of phospholipids in cell membranes. As part of energy-rich compounds such as nucleotides for instance phosphorus also plays an essential role in metabolism or its regulation. Myo-inositol-1,2,3,4,5,6-hexakisphosphate (IP6), also called phytate, was reported as the main storage form of phosphorus in cereals, legumes and nuts (Humer et al., 2015). However, phytate phosphorus is poorly available for monogastrics because of the low phytate dephosphorylating activity in their gastro-intestinal tract and the inability of phytate to be absorbed per se. Therefore, most of the phytate phosphorus is metabolized by the colon microflora or excreted and accumulates in soil. As a consequence strong effects on fluxes of phosphorus in the landscape and enrichment of water bodies with phosphorus have been reported resulting in eutrophication

*Abbreviations:* IPx, myo-inositol phosphate with x phosphate residues; M, maize; RM, rapeseed meal; SBM, soybean meal; SD, standard deviation; W, wheat.

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with many harmful environmental impacts such as shifts in species and variations in water quality and micro-organism composition (Correll, 1998; Smith et al., 1999). Phytate has also a strong antinutritional effect by forming indigestible complexes with nutrients, such as mineral divalent cations and proteins or dietary amino acids and may therefore increase their endogenous losses (Woyengo and Nyachoti, 2013). Thus, not only phosphorus has to be supplemented in diets as an inorganic feed additive in order to be bioavailable, but also other nutrients have to be incorporated at higher levels, both of which represent a significant cost in animal nutrition. Since their first commercialization in 1991, phytate-degrading enzymes, also called phytases, represent a widely recognized solution as feed additives by allowing phytate dephosphorylation during digestion (Lei and Stahl, 2000). Therefore, supplementation of phytase improves the bioavailability of both the organically bound phosphorus and the nutrients complexed by phytate.

Feed enzymes suppliers recommend a standard inclusion of 500 U of phytase per kg of feed in most pig and poultry feeds. However, differences in apparent activity of phytases were observed depending on the activity assay applied. In addition, the efficacy of phytases might also be altered by *in vivo* feed composition (Dersjant-Li et al., 2015). Therefore, phytases need to be studied in a model of the digestive tract under identical conditions to allow a proper comparison of their efficacies in the dephosphorylation of phytate. As *in vivo* evaluations of phosphorus uptake and assimilation are tedious, require a lot of time and repetitions, and are not always very sensitive and reproducible due to inner variations, *in vitro* models appear to be convenient alternatives (Shastak and Rodehutscord, 2013).

Conditions applied *in vitro* might be very different from those *in vivo*, therefore these models do not perfectly mimic the digestive process. However, they enable studying the enzymatic dephosphorylation of phytate under a broad variety of conditions more rapidly and more easily than in *in vivo* trials. A three-step *in vitro* model simulating the gastrointestinal tract of poultry was already described to investigate the dephosphorylation of phytate occurring in wheat in the presence of several phytases, taking temperature, retention time, pH, and proteolytic enzymes into account (Menezes-Blackburn et al., 2015). Therefore, this digestion model was adapted in order to study the enzymatic dephosphorylation of phytate from different feed ingredients or complete diets.

## 2. Materials and methods

### 2.1. Materials

The phytase used in this study was a recombinant enzyme from *Buttiauxella* expressed in *Trichoderma reesei* (Danisco, Copenhagen, Denmark). This solid phytase preparation was extracted (10% w/v) with sodium acetate buffer (20 mM, pH 5.5) for 2 h at 20 °C and centrifuged (25,000 *g*, 4 °C, 30 min) prior to characterization and use of the extract in the *in vitro* degradation model.

All feed ingredients (wheat, maize, soybean meal and rapeseed meal) and components of diets (inorganic salts, amino acids and premix) were ground to 0.5 mm using an ultra-centrifugal mill ZM 500 (Retsch®, Haan, Germany) with a 24-tooth rotor. Three complete diets were designed in order to mimic traditional poultry feeds (Table 1): a wheat-maize-soybean-meal-based diet (W.M.SBM), a maize-soybean-meal diet (M.SBM) and a wheat-maize-rapeseed-meal diet (W.M.RM). The initial phytate and inorganic phosphorus contents, along with the endogenous phytase activities of each feed ingredients and complete diets were measured prior to

**Table 1**
Composition (in g/kg) of the complete diets studied.

| | W.M.SBM[a] | M.SBM[a] | W.M.RM[a] |
|---|---|---|---|
| Wheat | 310.0 | – | 310.0 |
| Maize | 306.2 | 566.0 | 306.2 |
| Soybean meal | 250.0 | 300.0 | – |
| Rapeseed meal | – | – | 250.0 |
| Distiller's dried grains with solubles | 50.0 | 50.0 | 50.0 |
| Soybean oil | 25.0 | 25.0 | 25.0 |
| Sodium carbonate | 1.5 | 1.5 | 1.5 |
| Dicalcium phosphate | 8.0 | 8.0 | 8.0 |
| Calcium carbonate | 6.0 | 6.0 | 6.0 |
| Sodium chloride | 2.3 | 2.3 | 2.3 |
| D/L-Methionine | 16.0 | 16.0 | 16.0 |
| L-Lysine | 10.0 | 10.0 | 10.0 |
| L-Threonine | 6.0 | 6.0 | 6.0 |
| Premix[b] | 4.0 | 4.0 | 4.0 |
| Monensin | 5.0 | 5.0 | 5.0 |
| Total | 1000 | 1000 | 1000 |
| Calculated nutrient content | | | |
| - Metabolizable energy (kcal/kg) | 3009.0 | 3053.0 | 2943.0 |
| - Crude protein (g/kg) | 191.0 | 196.0 | 161.0 |
| - Ca (g/kg) | 7.9 | 8.0 | 2.6 |
| - Total P (g/kg) | 5.2 | 5.2 | 5.3 |
| - Available P (g/kg) | 2.5 | 2.8 | 3.5 |

[a] W.M.SBM, wheat-maize-soybean-meal-based diet; M.SBM, maize-soybean-meal diet; W.M.RM, wheat-maize-rapeseed-meal diet.
[b] SP7284, product supplied by CCPA (Janze, France). Composition (expressed per kg of feed): vitamin A (6000 UI), vitamin D3 (1200 UI), vitamin E (10 UI), vitamin B2 (2.50 mg), D-calcium panthotenate (5 mg), vitamin B12 (0.02 mg), niacinamide (13 mg), $FeSO_4 \cdot H_2O$ (80 mg), $CuSO_4 \cdot 5H_2O$ (13 mg), $ZnSO_4 \cdot H_2O$ (105 mg), MnO (40 mg), $Ca(IO_3)2$ (0.50 mg), $Na_2SeO_3$ (0.26 mg), sepiolite (4.30 mg) on calcium carbonate.

the use in the digestion simulation.

Phytic acid dodecasodium salt (Sigma 274321), pancreatin from porcine pancreas (4 × USP, Sigma P1750), and pepsin from porcine gastric mucosa (> 250 U/mg, Sigma P7125) were obtained from Sigma (Darmstadt, Germany). Ultrasep ES 100 RP18 column was purchased from Bischoff (Leonberg, Germany) and AG® 1-X4 anion exchange resin (100–200 mesh) from Bio-Rad (München, Germany). All reagents were of analytical grade.

### 2.2. Standard phytase activity assay

The microbial phytase activity assay was performed according to a previously described method (Greiner et al., 1993). Briefly, 10 µL of an appropriately diluted phytase solution was added to 350 µL of a 2.9 mM solution of phytic acid dodecasodium salt in sodium acetate buffer (100 mM, pH 5.5) and incubated at 37 °C. After a 30 min incubation, the released inorganic P was measured by using a modification of the ammonium molybdate method (Heinonen and Lahti, 1981): 1.5 mL of a stop solution of 10 mM ammonium heptamolybdate/2.5 M sulfuric acid/acetone (1:1:2 v/v/v) was added to the mixture, followed after 2 min by a 100 µL addition of a 1 M citric acid solution. The absorbance of the formed phospho-molybdate complex was measured at 355 nm. Prior to the phytase activity assay, the linearity of the phosphorus release from phytic acid dodecasodium salt was confirmed under the conditions used. P release was measured every five minutes during thirty minutes and the coefficient of determination of P release over time was higher than 0.99. Blanks were performed by adding the stop solution prior to enzyme addition. One unit of phytase activity was defined as the amount of phytase releasing 1 µmol of inorganic P in 1 min under test conditions.

### 2.3. Simulation of monogastrics' digestive tract

The simulation model was based on one previously described (Menezes-Blackburn et al., 2015). This three-step model was adapted into a monogastric digestion simulation. Step 1 (humidification) was performed by incubating 1 g of ground matrix (feed ingredient or complete diet) at 40 °C for 15 min with 6 mL of sodium acetate buffer (50 mM, pH 5.0) containing different amounts of phytase activity (0, 250, 500, 750, and 1000 U/kg feed). Step 2 (acidic treatment) was performed by adding to the previous digesta 560 µL of 1 M HCl and 1040 µL of sodium acetate buffer (50 mM, pH 3) containing 21 mg/mL of pepsin. The entire mixture was incubated at 40 °C for 10 min. Step 3 (alkaline treatment) was performed by adding to the previous digesta 1.3 mL of 1 M $NaHCO_3$ and 1.3 mL of 14.8 mg/mL aqueous pancreatin solution. The entire mixture was incubated at 40 °C for 15 min. All incubations were performed under continuous shaking to guarantee a homogeneous distribution of all ingredients.

The samples obtained from the simulated digestion were freeze-dried overnight (Christ, Osterode am Harz, Germany), and then extracted with 20 mL of 2.4% HCl for 3 h at room temperature. The resulting suspensions were centrifuged (30 min, 4 °C, 12,000 *g*) and the supernatants were collected and used for *myo*-inositol phosphate quantification (AOAC, 1990).

### 2.4. Quantification of myo-inositol phosphates

Two milliliters aliquots of the supernatants were diluted with 60 mL water. The entire solution was applied to a column (0.7 × 15 cm) containing 0.5 g of AG® 1-X4 anion exchange resin (100–200 mesh). The column was washed with 25 mL of water and 25 mL of 25 mM HCl. Then *myo*-inositol phosphates were eluted with 25 mL of 2 M HCl. The obtained eluates were concentrated in a vacuum evaporator and dissolved in 1 mL of water. Then 20 µL of the samples were analyzed by HPLC (Pharmacia, Uppsala, Sweden) on an Ultrasep ES 100 RP18 (2 × 250 mm) column using refractive index (RI) detection in order to quantify $IP_3$–$IP_6$ (PN 3150 RI Detector, Postnova Analytics, Landsberg am Lech, Germany). The elution was performed in isocratic mode at 40 °C with a 0.2 mL/min flow rate of solvent (formic acid/methanol/water/tetrabutylammonium hydroxide, 44:56:5:1.5 v/v, pH 4.25) (Sandberg and Ahderinne, 1986). A 1.67 mM aqueous solution of *myo*-inositol hexakisphosphate ester (IP6) was used as a standard.

### 2.5. Evaluation of feed ingredients and feeds prior to digestive process

In order to measure the inorganic phosphorus and phytate contents of feed ingredients and feeds, 1 g of matrix was extracted for 3 h with 20 mL of 2.4% HCl at 20 °C. The mixture was centrifuged (30 min, 4 °C, 12,000 *g*, Beckman, Krefeld, Germany) and the supernatants were collected and used for inorganic P and phytate quantification. IP3–IP6 were measured as described in Section 2.4. Inorganic phosphorus content was measured by using the modification of the ammonium molybdate method (Heinonen and Lahti, 1981) previously described: briefly, 1.5 mL of a stop solution of 10 mM ammonium heptamolybdate/2.5 M sulfuric acid/acetone (1:1:2 v/v/v) was added to 400 µL of supernatant, followed after 2 min by a 100 µL addition of a 1 M citric acid solution and the absorbance of the formed phospho-molybdate complex was measured at 355 nm.

The intrinsic phytase activity of feed ingredients and feeds was measured by direct incubation of 1 g in a 5 mM solution of sodium phytate in acetate buffer (100 mM, pH 5.5) for 30 min at 37 °C (Greiner and Egli, 2003). 50 µL of the mixture is sampled every 5 min and diluted with water, prior to measuring the released inorganic P as already described.

### 2.6. Calculation of phosphorus release from phytate

The amount of phosphorus released from IPx was calculated based on the *myo*-inositol phosphates (IP6–IP3) quantified in the feed ingredients and in the feed before and after *in vitro* digestion. Indeed, the values obtained by direct phosphate quantification were

higher than those obtained from phytate dephosphorylation. This might be due to a dephosphorylation of other phosphorylated compounds present in the feed or feed ingredients (Lowry and Lopez, 1946) or an interaction between the phosphate released and other constituents of the feeds or feed ingredients. The initial IP2 and IP1 contents of feed ingredients was reported insignificant (Pointillart, 1994) and therefore only their IP3–IP6 contents were considered. The phosphorus initially bound to the *myo*-inositol ring (IP3–IP6) could be calculated according to Eq. (1).

Calculation of initial phytate bound phosphorus (mole)

$$P_{phytate_{initial}} = \sum_{k=1}^{6} k * \mathrm{IP}_{k\,\mathrm{initial}} = \sum_{k=3}^{6} k * \mathrm{IP}_{k\,\mathrm{initial}} \quad (1)$$

where k is the number of P bound to the inositol ring. The HPLC system used is not capable of quantifying IP2, IP1 and *myo*-inositol and their quantification is of little interest, because IP4 and/or IP3 and not IP1 nor IP2 generally accumulate during digestion (Greiner, 2017; Schlemmer et al., 2001). After *in vitro* digestion however the concentrations of IP2, IP1 and *myo*-inositol need to be considered in order to calculate P released from the *myo*-inositol ring. Thus, the inositol amount calculated according to Eq. (2) before and after *in vitro* digestion and defined as (IP < 3) might either be present as *myo*-inositol, IP1 or IP2.

Material balance (mole) of inositol phosphates and calculation of IP < 3 content after digestion.

It is considered that

$$\sum_{k=1}^{6} IP_{k\,initial} = \sum_{k=3}^{6} IP_{k\,initial}$$

Thus,

$$\sum_{k=3}^{6} IP_{k\,initial} = \sum_{k=3}^{6} IP_{k\,final} + \sum_{k=1}^{2} IP_{k\,final} + myo\ \ inositol$$

It is defined

$$\sum_{k=1}^{2} IP_{k\,final} + \ myo\ \ inositol = (IP < 3)_{final}$$

Thus,

$$(IP < 3)_{final} = \sum_{k=3}^{6} IP_{k\,initial} - \sum_{k=3}^{6} IP_{k\,final} \quad (2)$$

Because the real-life situation will be in between the two extremes (100% as *myo*-inositol or 100% as IP2), the accumulation of IP1 exclusively was used as an intermediate situation to estimate the P released from the *myo*-inositol ring (Eq. (3)).

Material balance (mole) of originally phytate-bound phosphorus and calculation of P released after digestion.

$$\mathrm{Pphytate}_{\ initial} = \mathrm{Pphytate}_{\ final} + \mathrm{P}_{\ released}$$

With

$$Pphytate_{final} = \sum_{k=1}^{6} k * IP_{k\,final} = \sum_{k=3}^{6} k * IP_{k\,final} + \sum_{k=1}^{2} k * IP_{k\,final}$$

but

$$\sum_{k=1}^{2} k * IP_{k\,final} \quad is \quad unknown$$

Its highest value is $2 * \sum_{k=1}^{2} IP_{k\,final}$ and its lowest value is 0.

An accumulation of IP1 was considered, *i.e.* IP2 = 0 and *myo*-inositol = 0 as an intermediate situation

$$\sum_{k=1}^{2} k * IP_{k\,final} = \ 1 * \sum_{k=1}^{2} IP_{k\,final} = 1 * (IP < 3)_{final}$$

so

$$P_{released} \sim \sum_{k=3}^{6} k * IP_{k\,initial} - \sum_{k=3}^{6} k * IP_{k\,final} - 1 * (\sum_{k=3}^{6} IP_{k\,initial} - \sum_{k=3}^{6} IP_{k\,final}) \quad (3)$$

### 2.7. Data management

The statistical replicate of this work (n) is the calculation of P release based on the measured IP6–IP3 at the end of a laboratory run of digestion simulation for one ingredient and one phytase dose. Each experiment was performed multiple times. The initial phytate phosphorus content for each ingredient used to calculate P release for the different replicates were the average of at least 2 measurements but 3 for soybean meal, 5 for maize and 7 for wheat. Data were analyzed using R version 4.0.2 (R Core Team, 2020) by ANOVA to evaluate the impact of ingredients, phytase dose and their interactions on P liberation. Linear and quadratic effects of phytase dose were tested. Data with absolute values of standard residues above 3 were excluded. Rapeseed meal P release (in mg of P/kg of rapeseed meal) data had to be excluded from the ANOVA analysis because of their high variability which led to non-normal distribution of the residues (Shapiro-Wilk test). A Kruskal-Wallis test was performed on the initial IPx contents of feed ingredients and diets followed by a Dunn test for multiple pairwise comparisons. An effect was considered significant at P-values below 0.05 ($P \leq 0.05$).

## 3. Results

### 3.1. Phytate phosphorus content and endogenous phytase activity of the feed ingredients and complete diets

The range of phytate phosphorus content (1.8–9.8 g/kg) was wide among feed ingredients (Table 2). The values obtained for the complete diets agreed to those determined for the individual ingredients considering their relative proportions. Furthermore, huge differences in endogenous phytase activities was observed among the different feed ingredients. Wheat had a very high (> 1700 U/kg) endogenous phytase activity, higher than the highest phytase dose (1000 U/kg) added as a supplement to the *in vitro* digestion system. In maize and soybean meal however, the endogenous phytase activities were low (< 100 U/kg). An intermediate endogenous phytase activity (244 ± 124 U/kg) was found in rapeseed meal. The obtained phytate phosphorus contents and endogenous phytase activities of the feed ingredients are in good agreement with those already reported (Eeckhout and De Paepe, 1994; Selle et al., 2003; Viveros et al., 2000), except for rapeseed meal. The endogenous phytase activity for rapeseed meal was found to be higher than the published data. Moreover, in the complete diets the endogenous phytase activities obtained were higher than expected from the phytase activities of the single ingredients, although repetition of the measurements would be necessary to validate the results.

### 3.2. Myo-inositol phosphate hydrolysis during digestion simulations of feed ingredients

Differences in phytate dephosphorylation were observed with the different feed ingredients using the *in vitro* approach (Fig. 1). An almost complete IP6 dephosphorylation was obtained with wheat even without exogenous phytase addition. The high endogenous phytase activity of wheat could easily explain this observation. For maize and soybean meal, the lowest exogenous phytase dose tested (250 U/kg) was sufficient for a complete IP6 hydrolysis. Both feed ingredients exhibited a relatively low endogenous phytase activity but also a low phytate content compared to rapeseed meal. The initial contents of P bound to IPx significantly differed between feed ingredients ($P = 0.008$) with maize having a significantly lower content than rapeseed meal, the other feed ingredients being intermediate. Due to the high phytate content compared to the other feed ingredients a complete IP6 dephosphorylation was not achieved during *in vitro* digestion of rapeseed meal even at the highest dose (1000 U/kg) of exogenous phytase: about a 0.06 portion of the initial

**Table 2**
Phytate phosphorus content and endogenous phytase activities in feed ingredients and complete diets, compared to previously reported values in the literature (values are means of n measurements).

| | Present study | | | Values from the literature[b] | | |
|---|---|---|---|---|---|---|
| | Phytate P[a] (g/kg) | Inorganic P (g/kg) | Endogenous phytase (U/kg) | Phytate P[a] (g/kg) | Inorganic P (g/kg) | Endogenous phytase (U/kg) |
| Wheat | 2.1 ± 0.1 (n = 10) | 0.4 ± 0.01 (n = 2) | 1749 ± 71 (n = 2) | 1.3–3.2 | 0.6–1.1 | 255–1912 |
| Maize | 1.8 ± 0.1 (n = 9) | 0.3 ± 0.01 (n = 2) | 44 ± 5 (n = 2) | 1.6–2.6 | 0.3–0.5 | 0–75 |
| Soybean meal | 3.9 ± 0.2 (n = 5) | 1.0 ± 0.02 (n = 2) | 86 (n = 1) | 2.8–5.4 | 2.1–4.0 | 0–149 |
| Rapeseed meal | 9.8 ± 0.3 (n = 6) | 2.1 ± 0.01 (n = 2) | 244 ± 124 (n = 2) | 3.4–8.8 | 2.1–7.2 | 0–43 |
| W.M.SBM[c] | 2.6 ± 0.1 (n = 2) | 2.6 ± 0.1 (n = 2) | 883 (n = 1) | – | – | – |
| M.SBM[c] | 2.3 ± 0.1 (n = 2) | 2.5 ± 0.1 (n = 2) | 129 (n = 1) | – | – | – |
| W.M.RM[c] | 4.3 ± 0.2 (n = 2) | 3.3 ± 0.3 (n = 2) | 864 (n = 1) | – | – | – |

[a] IP3–IP6 bound phosphorus concentration.
[b] Eeckhout and De Paepe (1994), Selle et al. (2003) and Viveros et al. (2000).
[c] W.M.SBM, wheat-maize-soybean-meal-based diet; M.SBM, maize-soybean-meal diet; W.M.RM, wheat-maize-rapeseed-meal diet.

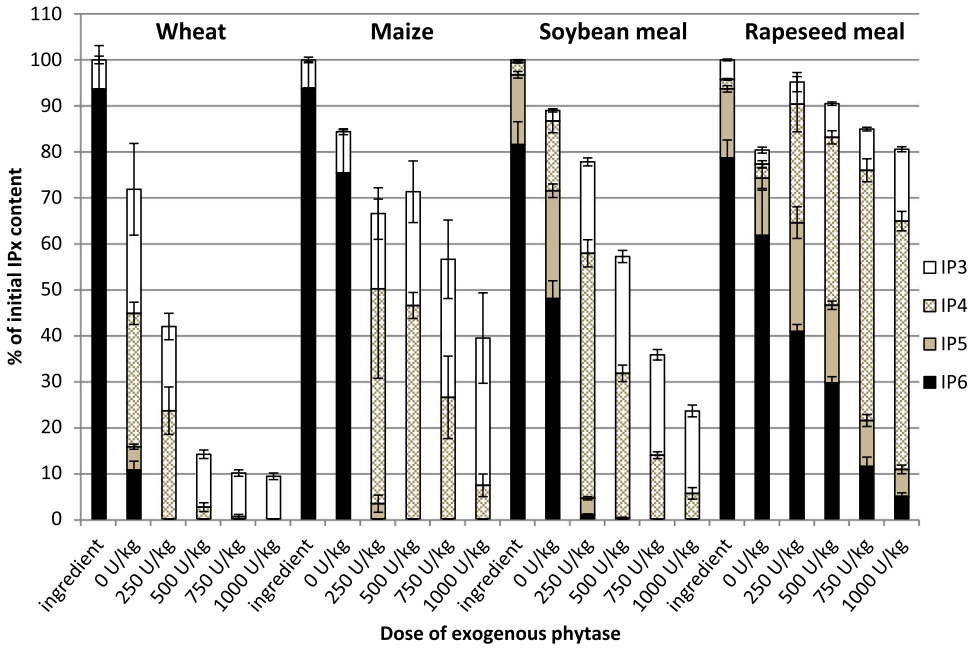


**Fig. 1.** Remaining phytate (IP6) and *myo*-inositol penta-, tetra- and triphosphate (IP5-3) arising from partial phytate hydrolysis in the digesta after *in vitro* simulations with the different feed ingredients, expressed in percentage of the initial concentrations of IPs. Error bars present the confidence interval of the mean.

IP6 content remained after digestion.

The profiles of the partially phosphorylated *myo*-inositol phosphates (IP5–IP3) generated during *in vitro* digestion were comparable among wheat, maize, soybean meal and rapeseed meal. IP5 didn't accumulate and was rapidly further dephosphorylated. The first phytate dephosphorylation product accumulating was IP4. After the complete disappearance of IP6, IP4 was further hydrolyzed to IP3 which accumulated later on.

The proportion of released phosphorus was found to be very different among the feed ingredients ($P < 0.001$, Table 3). Phosphorus release from phytate in wheat was high even without phytase addition (a 0.47 portion of its initial P content bound to IPx). This result was expected because of its very high endogenous phytase activity (1749 ± 71 U/kg). In comparison, phosphorus release from phytate in other ingredients only reached 0.15–0.19 portions of their initial P content bound to IPx. Addition of exogenous phytase increased the liberation of phosphorus by up to 1.7-fold in wheat at the highest dose (1000 U/kg) tested, resulting in the release of a 0.80 portion of the phosphorus initially bound to the *myo*-inositol ring. In the presence of 1000 U/kg of exogenous phytase, P release for maize reached 0.68 of the phosphorus initially bound to the *myo*-inositol ring, 0.74 for soybean meal and 0.40 for rapeseed meal.

### 3.3. *Myo-inositol phosphate hydrolysis during digestion simulations of feeds*

The three-step simulation process was also applied to three different feeds composed of the feed ingredients tested (Table 1): a wheat-maize-soybean-meal-based diet (W.M.SBM), a maize-soybean-meal diet (M.SBM) and a wheat-maize-rapeseed-meal diet (W.M.RM).

The sequential hydrolysis of IP6 were found to be similar among the three evaluated feeds (Fig. 2). Without addition of exogenous phytase, 0.75 (M.SBM), 0.54 (W.M.RM) and 0.39 (W.M.SBM) portions of the initial IP6 remained in the digesta of the *in vitro* simulation. The extent of phytate dephosphorylation is linked to the endogenous phytase activities (129, 864 and 883 U/kg for M.SBM, W.M.RM and W.M.SBM respectively) and initial IPx phosphorus content of the different diets (2.3, 4.3 and 2.6 g/kg).

Accumulation of the partially phosphorylated *myo*-inositol phosphates was in good agreement to IPx accumulation using the individual ingredients. No accumulation of IP5 was observed during *in vitro* digestion in the presence and absence of exogenous phytase, IP4 was the first intermediate accumulating and after the complete dephosphorylation of IP6, IP4 was further hydrolyzed to IP3. IP3 was also observed to accumulate.

P released is significantly increased in the presence of the highest dose (1000 U/kg) of exogenous phytase compared to the *in vitro* digestion without exogenous phytase ($P < 0.05$, Table 4): P release increased by a 3.3-fold (W.M.SBM), a 4.6-fold (M.SBM) and a 2.7-fold (W.M.RM). Phosphorus release from IPx was found to be increasing with increasing doses of exogenous phytase and more than a 0.6 portion of the phosphorus initially bound to the *myo*-inositol ring was released in the presence of 1000 U/kg exogenous phytase for all three diets studied. The amount of phosphorus released in the *in vitro* digestion model is dependent on the initial IPx phosphorus content of the feeds ($P < 0.001$). Feeds with higher initial phytate phosphorus concentration resulted in higher phosphorus releases although no significant difference between initial phytate contents was found between feeds ($P = 0.102$).

**Table 3**
Phosphorus released[a] (mg of phosphorus/kg of feed matrix and proportion of initial IPx phosphorus[b]) in digesta after *in vitro* simulations with different ingredients.

| Ingredient | Phytase dose (U/kg) | P released (mg/kg) | Proportion P released |
|---|---|---|---|
| Wheat (n = 26) | 0 | 997$^{fgh}$ | 0.47$^{qrst}$ |
| | 250 | 1353$^{hi}$ | 0.64$^{stuv}$ |
| | 500 | 1631$^{ij}$ | 0.78$^{vw}$ |
| | 750 | 1668$^{j}$ | 0.79$^{w}$ |
| | 1000 | 1675$^{j}$ | 0.80$^{w}$ |
| | SEM | 67 | 0.03 |
| Maize (n = 19) | 0 | 264$^{d}$ | 0.15$^{mn}$ |
| | 250 | 898$^{efg}$ | 0.51$^{pqrst}$ |
| | 500 | 891$^{ef}$ | 0.50$^{pqrs}$ |
| | 750 | 1041$^{efg}$ | 0.59$^{qrstu}$ |
| | 1000 | 1204$^{fgh}$ | 0.68$^{tuvw}$ |
| | SEM | 89 | 0.05 |
| Soybean meal (n = 17) | 0 | 751$^{e}$ | 0.19$^{mno}$ |
| | 250 | 1761$^{j}$ | 0.45$^{pqr}$ |
| | 500 | 2252$^{k}$ | 0.57$^{qrstu}$ |
| | 750 | 2669$^{l}$ | 0.68$^{tuvw}$ |
| | 1000 | 2892$^{l}$ | 0.74$^{uvw}$ |
| | SEM | 194 | 0.05 |
| Rapeseed meal[c] (n = 11) | 0 | 1619 | 0.17$^{mn}$ |
| | 250 | 1412 | 0.14$^{m}$ |
| | 500 | 2200 | 0.23$^{no}$ |
| | 750 | 3259 | 0.33$^{nop}$ |
| | 1000 | 3897 | 0.40$^{opq}$ |
| | SEM | 393 | 0.04 |
| Main effects: ingredient | | | |
| Wheat | | 1436 | 0.68 |
| Maize | | 858 | 0.48 |
| Soybean meal | | 1957 | 0.50 |
| Rapeseed meal | | 2399 | 0.25 |
| Phytase dose | | | |
| 0 | | 876 | 0.28 |
| 250 | | 1379 | 0.50 |
| 500 | | 1634 | 0.56 |
| 750 | | 1960 | 0.66 |
| 1000 | | 2091 | 0.68 |
| Significance (P-value)[c] | | | |
| Ingredient | | < 0.001 | < 0.001 |
| Phytase dose linear | | < 0.001 | < 0.001 |
| Phytase dose quadratic | | 0.034 | 0.051 |
| Ingredient*Phytase dose | | < 0.001 | 0.005 |

[a] Released P is estimated based on the intermediate situation of the accumulation of IP1 (Eq. (3)).
[b] Initial IPx phosphorus contents for proportion calculation are 2104 mg/kg for wheat, 1771 for maize, 3930 for soybean meal and 9773 for rapeseed meal.
[c] The absolute P release data for rapeseed meal were excluded from the ANOVA analysis because of their high variability.
[d–w] : Within column, means without common superscript differ ($P < 0.05$).

## 4. Discussion

Phytase efficacy is affected by many factors, among others the availability of phytate for enzymatic hydrolysis. Solubility of phytate during the digestive process, broadly reviewed for both poultry and pigs (Selle and Ravindran, 2007, 2008), is one of the factors influencing this availability, since only soluble phytate could act as a substrate for the phytases used as feed additives. Thus, phytate location within the feed matrix may also contribute to the availability of phytate for enzymatic hydrolysis. Phytate within the plant matrix needs to be released in order to be available for the exogenous phytases added to feeds. Phytate location in cereal and oilseeds was studied and it was found that distribution differs between grains (Erdman, 1979; O'Dell et al., 1972). In wheat and rapeseed, phytate is mainly distributed within the outer layer (aleurone layer and outer bran) of the grain, whereas the endosperm contains almost no phytate. Unlike other oilseeds, no specific site of phytate location was identified in soybean seed. For maize nearly 90% of its phytate phosphorus is present in the germ. Despite this wide variety of phytate locations among the tested feed ingredients and total diets, no significant differences in the relative appearance of partially phosphorylated *myo*-inositol phosphates (IP5 to IP3) were found in the *in vitro* digestion model: IP5 was quickly further dephosphorylated and IP4 was the first dephosphorylation product accumulating during IP6 hydrolysis followed by IP3. This result was expected, since the relative concentration of the different dephosphorylation products of phytate over time is mainly dependent on the phytase used and the viscosity of the system (Greiner, unpublished results). Only one single exogenous phytase was applied in the present study and the viscosities when using different feed ingredients or diets were similar.

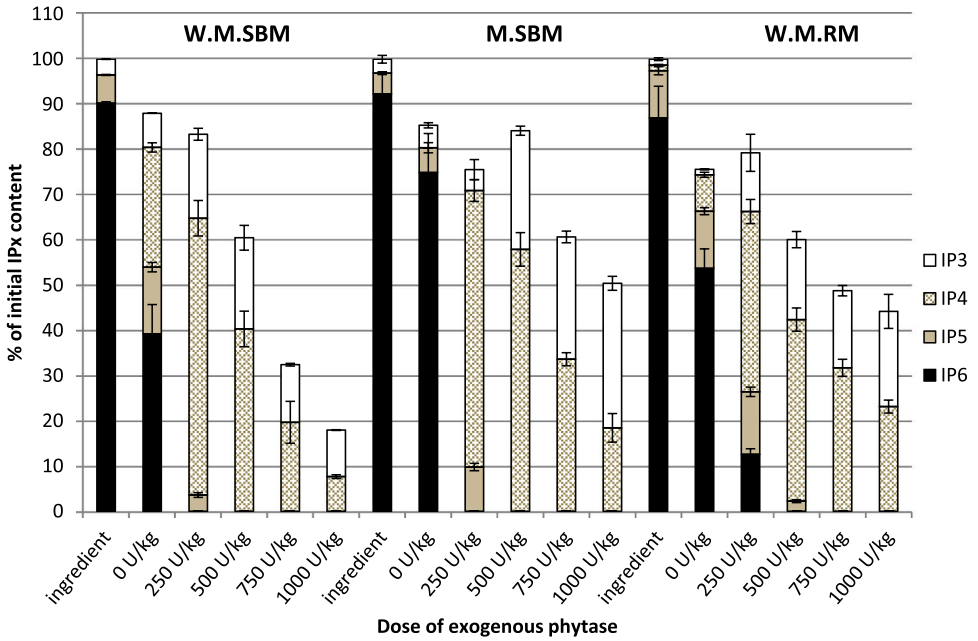


**Fig. 2.** Remaining phytate (IP6) and *myo*-inositol penta-, tetra- and triphosphate (IP5-3) arising from partial phytate hydrolysis in the digesta after *in vitro* simulations with the different feeds (W.M.SBM, wheat-maize-soybean-meal-based diet; M.SBM, maize-soybean-meal diet; W.M.RM, wheat-maize-rapeseed-meal diet), expressed in percentage of the initial concentrations of IPs. Error bars present the confidence interval of the mean.

The initial phytate phosphorus content seems to be a limiting factor of the enzymatic phytate dephosphorylation: the higher the initial phytate content, the more phytate is released into the liquid phase of the digestive tract in the model and therefore more phytate is available for the phytase to act on. Under *in vivo* conditions, when feeds are not ground, release of phytate from the feed matrix might be more pronounced than in the *in vitro* system depending on the location of the phytate within the matrix and the particle size of the feed particles. Furthermore, the higher viscosity under *in vivo* conditions might influence the release of phytate into the liquid phase and therefore the amount of phytate accessible to the phytase.

The concentration of phosphate may also affect enzymatic phytate dephosphorylation, because phosphate was reported to act as a competitive inhibitor for several phytases (Greiner et al., 1993). Assuming that the inhibition constants for the exogenous phytase used in this study is similar to those reported for the *E. coli* phytases (between 11.9 and 13 mM) (Greiner et al., 1993), the concentration of phosphate in the feed ingredients and diets is too low for an inhibition. During *in vitro* digestion however, the concentration of phosphate increased to a level where inhibition may occur.

As expected, the role of the intrinsic feed phytase in enzymatic phytate dephosphorylation was higher, the higher its level was but also the lower the activity of the added phytase was. Only for wheat exhibiting a much higher intrinsic phytase activity than the other feed ingredients, a noticeable role of the intrinsic phytase in enzymatic phytate dephosphorylation was observed. It is very likely that the intrinsic phytase dephosphorylates phytate in the feed matrix, because phytase and phytate are co-located within the feed matrix, whereas phytate need to be released from the feed matrix to be accessible to the exogenous phytase. Thus, the minor role of the intrinsic phytases might be explained by a fast release of phytate into the liquid phase of the *in vitro* system. It is also likely that the intrinsic plant phytases are more prone to inactivation under gastric and intestinal conditions compared to the added exogenous phytase (Selle and Ravindran, 2007, 2008). When incorporated to the feed for animal nutrition, these endogenous phytases are also prone to inactivation due to the fabrication process.

For the digestive process in the absence of exogenous phytase, the absolute P release depends both on the initial *myo*-inositol phosphate content and on the intrinsic phytase activity of the ingredient used (Tables 2 and 3). Some of the obtained values present a high variability and would need confirmation. It is especially the case for rapeseed meal which data had to be excluded from the analysis of variance. This high variability is probably partly due to experimental error, but a higher variability caused by the plant matrix cannot be excluded. Indeed, phytate is harder to extract from rapeseed meal than from soybean meal (Morgan et al., 2014). Despite this variability, some hypothesis can be made. The maize and soybean meal used in this study exhibited low intrinsic phytase activities (44 and 86 U/kg respectively) and therefore a low P release from the *myo*-inositol ring was observed (264 $\pm$ 271 and 751 $\pm$ 234 mg/kg respectively). The wheat used, with its significantly higher intrinsic phytase activity (1749 U/kg) resulted in a higher P release (997 $\pm$ 436 mg/kg) despite its low initial phytate content. A 0.46-fold decrease in its initial P content bound to the *myo*-inositol ring was observed. The rapeseed meal used had the highest initial content of *myo*-inositol-bound P (9.8 g/kg) and a high P release from IPx was obtained (1619 $\pm$ 1761 mg/kg). However, it represents only a 0.17-decrease of its initial *myo*-inositol-bound P content, because of its relatively low intrinsic phytase activity (244 U/kg). The same trends were also observed for the three complete diets (W.M.SBM, W.M.RM and M.SBM) used in this study.

**Table 4**
Phosphorus released[a] (mg of phosphorus/kg of feed matrix and proportion of initial IPx phosphorus[b]) in digesta after *in vitro* simulations with different feeds.

| Diet | Phytase dose (U/kg) | P released (mg/kg) | Proportion P released |
|---|---|---|---|
| W.M.SBM[c] (n = 10) | 0 | 605[d] | 0.23 |
| | 250 | 1124[ef] | 0.43 |
| | 500 | 1458[fgh] | 0.55 |
| | 750 | 1806[hi] | 0.68 |
| | 1000 | 1992[ji] | 0.75 |
| | SEM | 168 | 0.06 |
| M.SBM[c] (n = 10) | 0 | 309[d] | 0.14 |
| | 250 | 987[e] | 0.43 |
| | 500 | 1009[e] | 0.44 |
| | 750 | 1286[efg] | 0.56 |
| | 1000 | 1424[fg] | 0.63 |
| | SEM | 131 | 0.06 |
| W.M.RM[c] (n = 10) | 0 | 1014[e] | 0.24 |
| | 250 | 1619[gh] | 0.38 |
| | 500 | 2351[jk] | 0.55 |
| | 750 | 2613[kl] | 0.61 |
| | 1000 | 2744[l] | 0.64 |
| | SEM | 220 | 0.05 |
| Main effects: Diet | | | |
| W.M.SBM | | 1397 | 0.53[n] |
| M.SBM | | 1003 | 0.44[m] |
| W.M.RM | | 2068 | 0.48[mn] |
| Phytase dose | | | |
| 0 | | 643 | 0.20[w] |
| 250 | | 1243 | 0.41[x] |
| 500 | | 1606 | 0.51[y] |
| 750 | | 1901 | 0.62[z] |
| 1000 | | 2053 | 0.67[z] |
| Significance (P-value) | | | |
| Diet | | < 0.001 | 0.002 |
| Phytase dose linear | | < 0.001 | < 0.001 |
| Phytase dose quadratic | | 0.021 | 0.007 |
| Diet * Phytase dose | | 0.005 | 0.162 |

[a] Released P is estimated based on the intermediate situation of the accumulation of IP1 (Eq. (3)).
[b] Initial IPx phosphorus contents for proportion calculation are 2642 mg/kg for W.M.SBM, 2275 for M.SBM and 4306 for W.M.RM.
[c] W.M.SBM, wheat-maize-soybean-meal-based diet; M.SBM, maize-soybean-meal diet; W.M.RM, wheat-maize-rapeseed-meal diet.
[d–l] : Within column, means without common superscript differ ($P < 0.05$).
[m–n] : Within diet, means without common superscript differ ($P < 0.05$).
[w–z] : Within phytase dose, means without common superscript differ ($P < 0.05$).

The intrinsic phytase activities determined for the different diets are always higher than the sum of the intrinsic phytase activities of their individual ingredients (Table 2). A similar phenomenon was observed when mixing pea flour with wheat flour (Chouchene et al., 2018). A synergistic effect between the pea and wheat phytases was given as an explanation for the observed effect. Considering the high phytate concentration relative to the intrinsic phytase content in the phytase activity assay and the short time of incubation, a synergistic effect is very unlikely. During the assay at maximum a 0.03 part of the P bound to phytate was released and therefore the phytases present in the mixture only act on IP6 and therefore independently. Either a compound present in one ingredient acting as activator of the intrinsic phytase of another ingredient or phytase inhibitors present in the different ingredients being inactivated in the diets might explain the observation.

## 5. Conclusion

An *in vitro* digestive simulation process was a successful tool to assess the efficacy of a bacterial phytase used as a feed supplement in order to dephosphorylate phytate present in different feed ingredients and complete diets. Phytase efficacy was dependent on the feed or feed ingredient, partially due to their initial amount of phytate. The intrinsic plant-derived phytase had only a measurable role in phytate dephosphorylation when its activity was high such as in wheat and low doses of exogenous bacterial phytase was added. In the *in vitro* system, feed ingredients were ground and other elements might have a greater influence under *in vivo* conditions: release of phytate from the feed matrix might vary depending on the location of the phytate within the matrix, the particle size of the feed particles or the viscosity of the digesta. In conclusion, the *in vitro* digestion model applied was proven to be a simple and useful tool to assess the enzymatic dephosphorylation of phytate during digestion with different parameters and could be used to study specific parameters affecting the enzymatic dephosphorylation of phytate under digestive conditions.

## CRediT authorship contribution statement

**A. Riviere:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **T. Nothof:** Investigation. **R. Greiner:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Supervision. **S. Tranchimand:** Conceptualization, Methodology, Validation, Writing – review & editing. **N. Noiret:** Supervision, Project administration. **F. Robert:** Supervision, Project administration, Funding acquisition. **M. Mireaux:** Conceptualization, Methodology, Validation, Writing – review & editing, Project administration.

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## Declaration of Competing Interest

The authors confirm that there are not conflicts of interest in this research.

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