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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.

Keywords: *in vitro* digestion model, phytate, phytase, phosphorus, feed ingredient

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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58 **1. Introduction**

59 Phosphorus (P) is an essential nutrient in animal nutrition. It has an important structural role in
60 bone mineralization, as a component of the crystalline structure of the skeleton, as well as in the
61 form of phospholipids in cell membranes. As part of energy-rich compounds such as nucleotides
62 for instance phosphorus also plays an essential role in metabolism or its regulation. *Myo*-inositol-
63 1,2,3,4,5,6-hexakisphosphate (IP6), also called phytate, was reported as the main storage form of
64 phosphorus in cereals, legumes and nuts (Humer et al., 2015). However, phytate phosphorus is
65 poorly available for monogastrics because of the low phytate dephosphorylating activity in their
66 gastro-intestinal tract and the inability of phytate to be absorbed per se. Therefore, most of the
67 phytate phosphorus is metabolized by the colon microflora or excreted and accumulates in soil. As
68 a consequence strong effects on fluxes of phosphorus in the landscape and enrichment of water
69 bodies with phosphorus have been reported resulting in eutrophication with many harmful
70 environmental impacts such as shifts in species and variations in water quality and micro-organism
71 composition (Correll, 1998; Smith et al., 1999). Phytate has also a strong antinutritional effect by
72 forming indigestible complexes with nutrients, such as mineral divalent cations and proteins or
73 dietary amino acids and may therefore increase their endogenous losses (Woyengo and Nyachoti,
74 2013). Thus, not only phosphorus has to be supplemented in diets as an inorganic feed additive in
75 order to be bioavailable, but also other nutrients have to be incorporated at higher levels, both of
76 which represent a significant cost in animal nutrition. Since their first commercialization in 1991,
77 phytate-degrading enzymes, also called phytases, represent a widely recognized solution as feed
78 additives by allowing phytate dephosphorylation during digestion (Lei and Stahl, 2000).
79 Therefore, supplementation of phytase improves the bioavailability of both the organically bound
80 phosphorus and the nutrients complexed by phytate.

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

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

99

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100 **2. Materials and methods**

101 *2.1. Materials*

102 The phytase used in this study was a recombinant enzyme from *Buttiauxella* expressed in
103 *Trichoderma reesei* (Danisco, Copenhagen, Denmark). This solid phytase preparation was
104 extracted (10% w/v) with sodium acetate buffer (20 mM, pH 5.5) for 2 h at 20 °C and centrifuged
105 (25000*g*, 4 °C, 30 min) prior to characterization and use of the extract in the *in vitro* degradation
106 model.

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

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

120 *2.2. Standard phytase activity assay*

121 The microbial phytase activity assay was performed according to a previously described
122 method (Greiner et al., 1993). Briefly, 10 μL of an appropriately diluted phytase solution was

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123 added to 350 µL of a 2.9 mM solution of phytic acid dodecasodium salt in sodium acetate buffer
124 (100 mM, pH 5.5) and incubated at 37 °C. After a 30 min incubation, the released inorganic P was
125 measured by using a modification of the ammonium molybdate method (Heinonen and Lahti,
126 1981): 1.5 mL of a stop solution of 10 mM ammonium heptamolybdate / 2.5 M sulfuric acid /
127 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
128 citric acid solution. The absorbance of the formed phospho-molybdate complex was measured at
129 355 nm. Prior to the phytase activity assay, the linearity of the phosphorus release from phytic acid
130 dodecasodium salt was confirmed under the conditions used
131 . P release was measured every five minutes during thirty minutes
132 and the coefficient of determination of P release over time was higher than 0.99. Blanks were
133 performed by adding the stop solution prior to enzyme addition. One unit of phytase activity was
134 defined as the amount of phytase releasing 1 µmol of inorganic P in 1 min under test conditions.
135 *2.3. Simulation of monogastrics' digestive tract*
136 The simulation model was based on one previously described (Menezes-Blackburn et al.,
137 2015). This three-step model was adapted into a monogastric digestion simulation. Step 1
138 (humidification) was performed by incubating 1 g of ground matrix (feed ingredient or complete
139 diet) at 40 °C for 15 min with 6 mL of sodium acetate buffer (50 mM, pH 5.0) containing different
140 amounts of phytase activity (0, 250, 500, 750, and 1000 U/kg feed). Step 2 (acidic treatment) was
141 performed by adding to the previous digesta 560 µL of 1 M HCl and 1040 µL of sodium acetate
142 buffer (50 mM, pH 3) containing 21 mg/mL of pepsin. The entire mixture was incubated at 40 °C
143 for 10 min. Step 3 (alkaline treatment) was performed by adding to the previous digesta 1.3 mL of
144 1 M $NaHCO_3$ and 1.3 mL of 14.8 mg/mL aqueous pancreatin solution. The entire mixture was

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145 incubated at 40 °C for 15 min. All incubations were performed under continuous shaking to
146 guarantee a homogeneous distribution of all ingredients.

147 The samples obtained from the simulated digestion were freeze-dried overnight (Christ,
148 Osterode am Harz, Germany), and then extracted with 20 mL of 2.4 % HCl for 3 h at room
149 temperature. The resulting suspensions were centrifuged (30 min, 4 °C, 12000*g*) and the
150 supernatants were collected and used for *myo*-inositol phosphate quantification (AOAC Method
151 986.11, 1990).

152 *2.4. Quantification of myo-inositol phosphates*

153 Two milliliters aliquots of the supernatants were diluted with 60 mL water. The entire solution
154 was applied to a column (0.7 × 15 cm) containing 0.5 g of AG® 1-X4 anion exchange resin (100–
155 200 mesh). The column was washed with 25 mL of water and 25 mL of 25 mM HCl. Then *myo*-
156 inositol phosphates were eluted with 25 mL of 2 M HCl. The obtained eluates were concentrated
157 in a vacuum evaporator and dissolved in 1 mL of water. Then 20 µL of the samples were analyzed
158 by HPLC (Pharmacia, Uppsala, Sweden) on an Ultrasep ES 100 RP18 (2 × 250 mm) column using
159 refractive index (RI) detection in order to quantify IP_3 to IP_6. (PN 3150 RI Detector, Postnova
160 Analytics, Landsberg am Lech, Germany). The elution was performed in isocratic mode at 40 °C
161 with a 0.2 mL/min flow rate of solvent (formic acid / methanol / water / tetrabutylammonium
162 hydroxide, 44:56:5:1.5 v/v, pH 4.25) (Sandberg and Ahderinne, 1986). A 1.67 mM aqueous
163 solution of *myo*-inositol hexakisphosphate ester (IP6) was used as a standard.

164 *2.5. Evaluation of feed ingredients and feeds prior to digestive process*

165 In order to measure the inorganic phosphorus and phytate contents of feed ingredients and
166 feeds, 1 g of matrix was extracted for 3 h with 20 mL of 2.4 % HCl at 20 °C. The mixture was
167 centrifuged (30 min, 4°C, 12000*g*, Beckman, Krefeld, Germany) and the supernatants were

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168 collected and used for inorganic P and phytate quantification. IP3 to IP6 were measured as
169 described in 2.4. Inorganic phosphorus content was measured by using the modification of the
170 ammonium molybdate method (Heinonen and Lahti, 1981) previously described: briefly, 1.5 mL
171 of a stop solution of 10 mM ammonium heptamolybdate / 2.5 M sulfuric acid / acetone (1:1:2 v/v/v)
172 was added to 400 µL of supernatant, followed after 2 min by a 100 µL addition of a 1 M citric acid
173 solution and the absorbance of the formed phospho-molybdate complex was measured at 355 nm.
174 The intrinsic phytase activity of feed ingredients and feeds was measured by direct incubation
175 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
176 (Greiner and Egli, 2003). 50 µL of the mixture is sampled every 5 min and diluted with water,
177 prior to measuring the released inorganic P as already described.

178 *2.6. Calculation of phosphorus release from phytate*

179 The amount of phosphorus released from IPx was calculated based on the *myo*-inositol
180 phosphates (IP6 – IP3) quantified in the feed ingredients and in the feed before and after *in vitro*
181 digestion. Indeed, the values obtained by direct phosphate quantification were higher than those
182 obtained from phytate dephosphorylation. This might be due to a dephosphorylation of other
183 phosphorylated compounds present in the feed or feed ingredients (Lowry and Lopez, 1946) or an
184 interaction between the phosphate released and other constituents of the feeds or feed ingredients.
185 The initial IP2 and IP1 contents of feed ingredients was reported insignificant (Pointillart, 1994)
186 and therefore only their IP3 to IP6 contents were considered. The phosphorus initially bound to
187 the *myo*-inositol ring (IP3 to IP6) could be calculated according to Equation 1.

188

189 Equation 1

190 Calculation of initial phytate bound phosphorus (mole)

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191 $$P_{phytate_{initial}} = \sum_{k=1}^{6} k * IP_{k_{initial}} = \sum_{k=3}^{6} k * IP_{k_{initial}}$$

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

199

200 Equation 2

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

202 It is considered that

203 $$\sum_{k=1}^{6} IP_{k_{initial}} = \sum_{k=3}^{6} IP_{k_{initial}}$$

204 Thus,

205 $$\sum_{k=3}^{6} IP_{k_{initial}} = \sum_{k=3}^{6} IP_{k_{final}} + \sum_{k=1}^{2} IP_{k_{final}} + myo\ inositol$$

206 It is defined

207 $$\sum_{k=1}^{2} IP_{k_{final}} + myo\ inositol = (IP < 3)_{final}$$

208 Thus,

209 $$(IP < 3)_{final} = \sum_{k=3}^{6} IP_{k_{initial}} - \sum_{k=3}^{6} IP_{k_{final}}$$

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210

211 Because the real-life situation will be in between the two extremes (100 % as *myo*-inositol or
212 100 % as IP2), the accumulation of IP1 exclusively was used as an intermediate situation to
213 estimate the P released from the *myo*-inositol ring. (Equation 3)

214

215 Equation 3

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

218 $\text{Pphytate}_{initial} = \text{Pphytate}_{final} + \text{P}_{released}$

219 With

220 $$Pphytate_{final} = \sum_{k=1}^{6} k * IP_{k_{final}} = \sum_{k=3}^{6} k * IP_{k_{final}} + \sum_{k=1}^{2} k * IP_{k_{final}}$$

221 but

222 $$\sum_{k=1}^{2} k * IP_{k_{final}} \quad is\ unknown$$

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

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

226 $$\sum_{k=1}^{2} k * IP_{k_{final}} = 1 * \sum_{k=1}^{2} IP_{k_{final}} = 1 * (IP < 3)_{final}$$

227 SO

228 $$P_{released} \sim \sum_{k=3}^{6} k * IP_{k_{initial}} - \sum_{k=3}^{6} k * IP_{k_{final}} - 1 * (\sum_{k=3}^{6} IP_{k_{initial}} - \sum_{k=3}^{6} IP_{k_{final}})$$

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229 *2.7. Data management*

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

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243 **3. Results**

244 *3.1 Phytate phosphorus content and endogenous phytase activity of the feed ingredients and*
245 *complete diets.*

246 The range of phytate phosphorus content (1.8 to 9.8 g/kg) was wide among feed ingredients
247 (Table 2). The values obtained for the complete diets agreed to those determined for the individual
248 ingredients considering their relative proportions. Furthermore, huge differences in endogenous
249 phytase activities was observed among the different feed ingredients. Wheat had a very high
250 (>1700 U/kg) endogenous phytase activity, higher than the highest phytase dose (1000 U/kg)
251 added as a supplement to the *in vitro* digestion system. In maize and soybean meal however, the
252 endogenous phytase activities were low (<100 U/kg). An intermediate endogenous phytase activity
253 (244 ± 124 U/kg) was found in rapeseed meal. The obtained phytate phosphorus contents and
254 endogenous phytase activities of the feed ingredients are in good agreement with those already
255 reported (Eeckhout and De Paepe, 1994; Selle et al., 2003; Viveros et al., 2000), except for
256 rapeseed meal. The endogenous phytase activity for rapeseed meal was found to be higher than
257 the published data. Moreover, in the complete diets the endogenous phytase activities obtained
258 were higher than expected from the phytase activities of the single ingredients, although repetition
259 of the measurements would be necessary to validate the results.

260 *3.2 Myo-inositol phosphate hydrolysis during digestion simulations of feed ingredients*

261 Differences in phytate dephosphorylation were observed with the different feed ingredients
262 using the *in vitro* approach (Figure 1). An almost complete IP6 dephosphorylation was obtained
263 with wheat even without exogenous phytase addition. The high endogenous phytase activity of
264 wheat could easily explain this observation. For maize and soybean meal, the lowest exogenous
265 phytase dose tested (250 U/kg) was sufficient for a complete IP6 hydrolysis. Both feed ingredients

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266 exhibited a relatively low endogenous phytase activity but also a low phytate content compared to
267 rapeseed meal. The initial contents of P bound to IPx significantly differed between feed
268 ingredients (p=0.008) with maize having a significantly lower content than rapeseed meal, the
269 other feed ingredients being intermediate. Due to the high phytate content compared to the other
270 feed ingredients a complete IP6 dephosphorylation was not achieved during *in vitro* digestion of
271 rapeseed meal even at the highest dose (1000 U/kg) of exogenous phytase: about a 0.06 portion of
272 the initial IP6 content remained after digestion.

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

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

288 *3.3. Myo-inositol phosphate hydrolysis during digestion simulations of feeds*

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289 The three-step simulation process was also applied to three different feeds composed of the feed
290 ingredients tested (Table 1): a wheat-maize-soybean-meal-based diet (W.M.SBM), a maize-
291 soybean-meal diet (M.SBM) and a wheat-maize-rapeseed-meal diet (W.M.RM).
292 The sequential hydrolysis of IP6 were found to be similar among the three evaluated feeds
293 (Figure 2). Without addition of exogenous phytase, 0.75 (M.SBM), 0.54 (W.M.RM) and 0.39
294 (W.M.SBM) portions of the initial IP6 remained in the digesta of the *in vitro* simulation. The extent
295 of phytate dephosphorylation is linked to the endogenous phytase activities (129, 864 and 883
296 U/kg for M.SBM, W.M.RM and W.M.SBM respectively) and initial IPx phosphorus content of
297 the different diets (2.3, 4.3 and 2.6 g/kg).
298 Accumulation of the partially phosphorylated *myo*-inositol phosphates was in good agreement
299 to IPx accumulation using the individual ingredients. No accumulation of IP5 was observed during
300 *in vitro* digestion in the presence and absence of exogenous phytase, IP4 was the first intermediate
301 accumulating and after the complete dephosphorylation of IP6, IP4 was further hydrolyzed to IP3.
302 IP3 was also observed to accumulate.
303 P released is significantly increased in the presence of the highest dose (1000 U/kg) of
304 exogenous phytase compared to the *in vitro* digestion without exogenous phytase ($P<0.05$, Table
305 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).
306 Phosphorus release from IPx was found to be increasing with increasing doses of exogenous
307 phytase and more than a 0.6 portion of the phosphorus initially bound to the *myo*-inositol ring was
308 released in the presence of 1000 U/kg exogenous phytase for all three diets studied. The amount
309 of phosphorus released in the *in vitro* digestion model is dependent on the initial IPx phosphorus
310 content of the feeds ($P<0.001$). Feeds with higher initial phytate phosphorus concentration resulted

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311 in higher phosphorus releases although no significant difference between initial phytate contents

312 was found between feeds (p=0.102).

313

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314 **4. Discussion**

315 Phytase efficacy is affected by many factors, among others the availability of phytate for
316 enzymatic hydrolysis. Solubility of phytate during the digestive process, broadly reviewed for both
317 poultry and pigs (Selle and Ravindran, 2008, 2007), is one of the factors influencing this
318 availability, since only soluble phytate could act as a substrate for the phytases used as feed
319 additives. Thus, phytate location within the feed matrix may also contribute to the availability of
320 phytate for enzymatic hydrolysis. Phytate within the plant matrix needs to be released in order to
321 be available for the exogenous phytases added to feeds. Phytate location in cereal and oilseeds was
322 studied and it was found that distribution differs between grains (Erdman, 1979; O'Dell et al.,
323 1972). In wheat and rapeseed, phytate is mainly distributed within the outer layer (aleurone layer
324 and outer bran) of the grain, whereas the endosperm contains almost no phytate. Unlike other
325 oilseeds, no specific site of phytate location was identified in soybean seed. For maize
326 nearly 90 % of its phytate phosphorus is present in the germ. Despite this wide variety of phytate
327 locations among the tested feed ingredients and total diets, no significant differences in the relative
328 appearance of partially phosphorylated *myo*-inositol phosphates (IP5 to IP3) were found in the *in*
329 *vitro* digestion model: IP5 was quickly further dephosphorylated and IP4 was the first
330 dephosphorylation product accumulating during IP6 hydrolysis followed by IP3. This result was
331 expected, since the relative concentration of the different dephosphorylation products of phytate
332 over time is mainly dependent on the phytase used and the viscosity of the system (Greiner,
333 unpublished results). Only one single exogenous phytase was applied in the present study and the
334 viscosities when using different feed ingredients or diets were similar.

335 The initial phytate phosphorus content seems to be a limiting factor of the enzymatic phytate
336 dephosphorylation: the higher the initial phytate content, the more phytate is released into the

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337 liquid phase of the digestive tract in the model and therefore more phytate is available for the
338 phytase to act on. Under *in vivo* conditions, when feeds are not ground, release of phytate from
339 the feed matrix might be more pronounced than in the *in vitro* system depending on the location
340 of the phytate within the matrix and the particle size of the feed particles. Furthermore, the higher
341 viscosity under *in vivo* conditions might influence the release of phytate into the liquid phase and
342 therefore the amount of phytate accessible to the phytase.

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

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

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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 (Table 2-3). 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 ± 271 and 751 ± 234 mg/kg respectively). The wheat used, with its significantly higher intrinsic phytase activity (1749 U/kg) resulted in a higher P release (997 ± 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 ± 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.

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

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383 effect. Considering the high phytate concentration relative to the intrinsic phytase content in the
384 phytase activity assay and the short time of incubation, a synergistic effect is very unlikely. During
385 the assay at maximum a 0.03 part of the P bound to phytate was released and therefore the phytases
386 present in the mixture only act on IP6 and therefore independently. Either a compound present in
387 one ingredient acting as activator of the intrinsic phytase of another ingredient or phytase inhibitors
388 present in the different ingredients being inactivated in the diets might explain the observation.

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389 **5. Conclusion**

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

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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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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 ~~(W.M.SBM)~~; M.SBM, maize-soybean-meal diet ~~(M.SBM)~~; W.M.RM, wheat-maize-rapeseed-meal diet ~~(W.M.RM)~~

[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.

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

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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	0	997[efgh]	0.47[pqrst]
(n =26)	250	1353[ghi]	0.64[rstuv]
	500	1631[hij]	0.78[uvw]
	750	1668[ij]	0.79[wx]
	1000	1675[ij]	0.80[wx]
	SEM	67	0.03
Maize	0	264[ed]	0.15[lmn]
(n =19)	250	898[defg]	0.51[opqrst]
	500	891[def]	0.50[opqrs]
	750	1041[defg]	0.59[pqrstu]
	1000	1204[efgh]	0.68[stuvw]
	SEM	89	0.05
Soybean meal	0	751[ed]	0.19[lmno]
(n =17)	250	1761[ij]	0.45[opqr]
	500	2252[kj]	0.57[pqrstu]
	750	2669[lk]	0.68[stuvw]
	1000	2892[lk]	0.74[uvw]
	SEM	194	0.05
Rapeseed meal[cb]	0	1619	0.17[lmn]
(n =11)	250	1412	0.14[ml]
	500	2200	0.23[mno]
	750	3259	0.33[mnop]
	1000	3897	0.40[nopq]
	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)[bc]			
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 (Equation 3).

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[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)

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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[de]	0.23
	250	1124[defg]	0.43
	500	1458[efgh]	0.55
	750	1806[ghi]	0.68
	1000	1992[hji]	0.75
	SEM	168	0.06
M.SBM[c] (n =10)	0	309[de]	0.14
	250	987[ed]	0.43
	500	1009[ed]	0.44
	750	1286[defg]	0.56
	1000	1424[efg]	0.63
	SEM	131	0.06
W.M.RM[c] (n =10)	0	1014[ed]	0.24
	250	1619[fgh]	0.38
	500	2351[ijk]	0.55
	750	2613[jkl]	0.61
	1000	2744[lk]	0.64
	SEM	220	0.05
Main effects: Diet			
W.M.SBM		1397	0.53[nm]
M.SBM		1003	0.44[ml]
W.M.RM		2068	0.48[lmn]
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 (Equation 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 (W.M.SBM); M.SBM, maize-soybean-meal diet (M.SBM); W.M.RM, wheat-maize-rapeseed-meal diet (W.M.RM)

[ed-kl]: Within column, means without common superscript differ (P<0.05)

[ml-nm]: Within diet, means without common superscript differ (P<0.05)

[w-z]: Within phytase dose, means without common superscript differ (P<0.05)

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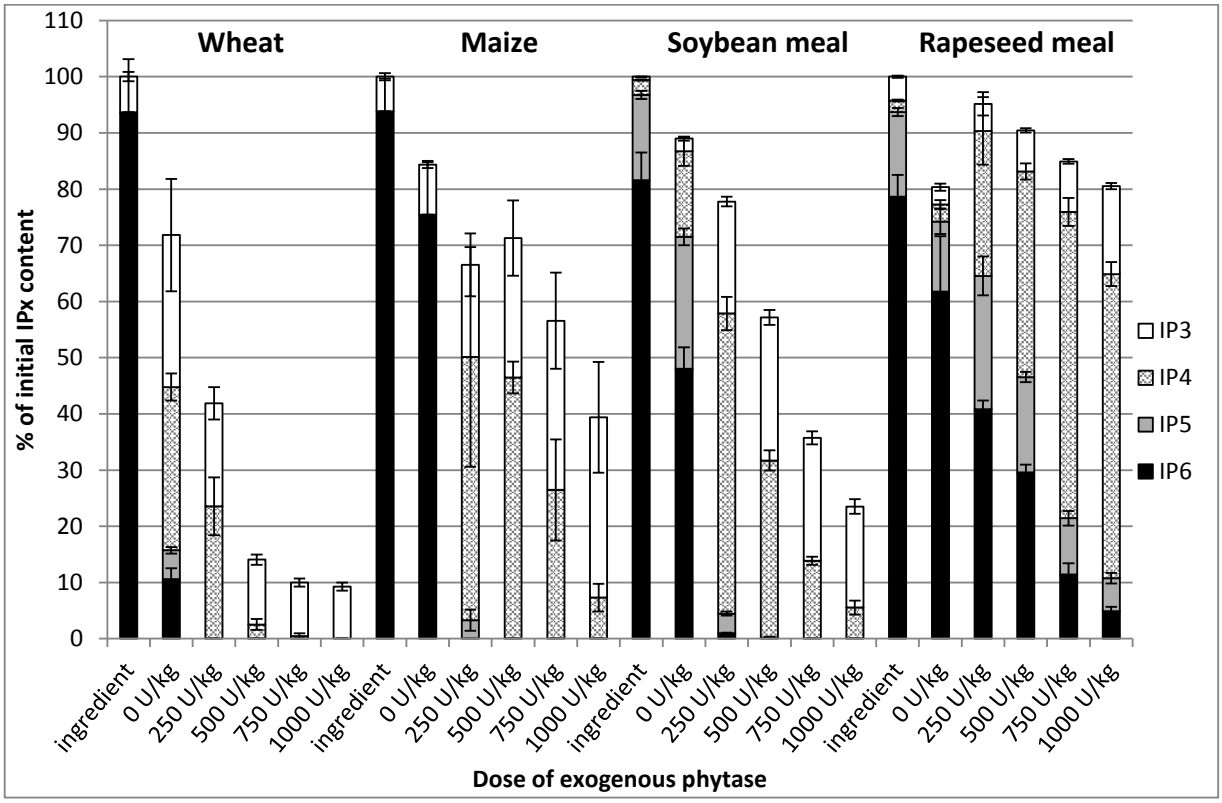

Figure 1

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.

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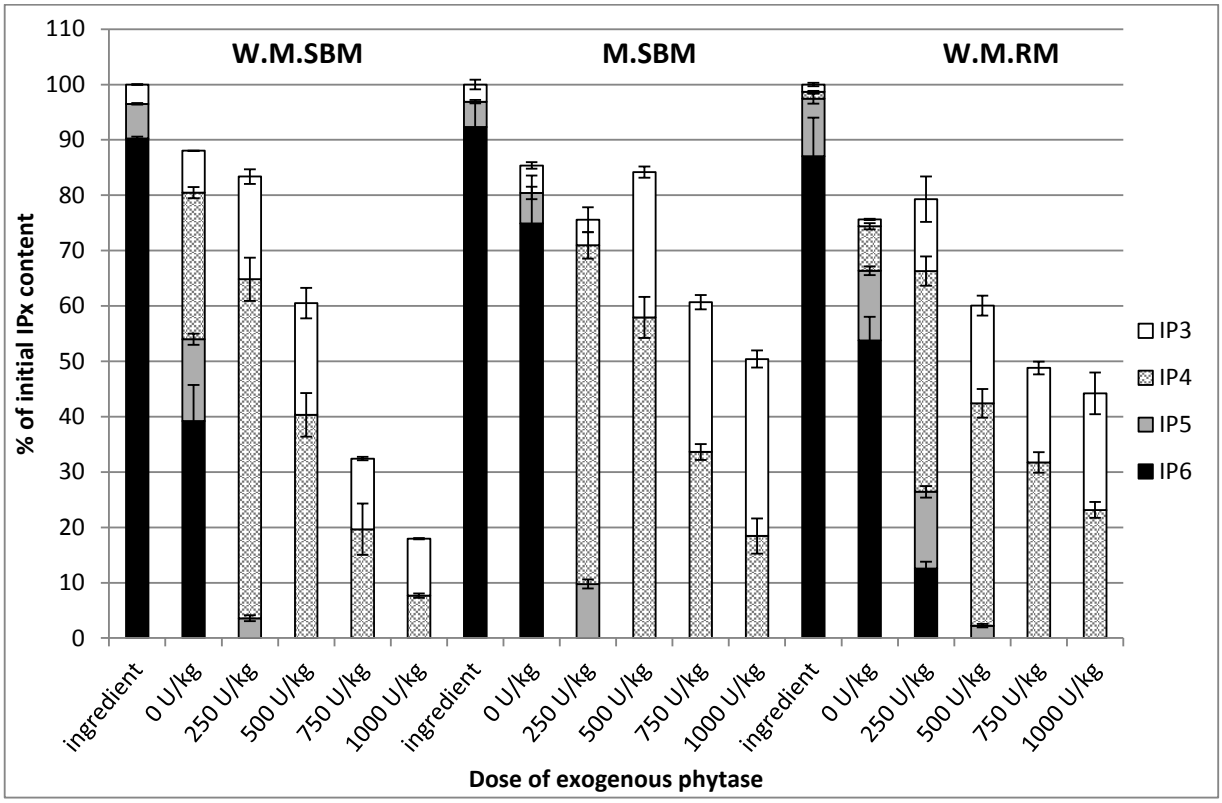

Figure 2

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.

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