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Effects of high moisture airtight storage of barley with exogenous enzymes on phosphorus digestibility of barley fed to pigs alone or in combination with soybean meal

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ABSTRACT

The aim was to study the effect of storage (high moisture airtight (HMA) storage vs. dry storage) and a combination of phytase, xylanase, β-glucanase, and protease on nutrient solubility and digestibility of barley. Experiment 1 studied the effect of HMA stored barley at 29%, 35%, and 40% moisture and enzyme combination to define the optimum moisture level for the maximum nutrient solubility of barley. The '35% moisture' was chosen for HMA storage of barley because this treatment (without or with enzymes) enhanced phytate degradation (31–73%) and the solubility of P (125–177%), nitrogen (N) (95–125%) and protein (56–68%) compared to dry storage ($P < 0.05$) and at the same time seemed to prevent a loss of energy and N (ammonia) by microbial fermentation. In Exp. 2, 6 female growing pigs (43 ± 2 kg) were used per diet (8 diets, 48 pigs) in a 12-d nutrient digestibility study to assess the effect of HMA stored barley at 35% moisture and enzyme combination on coefficient of total tract apparent digestibility (CTTAD) of P in barley fed alone or together with soybean meal (SBM). Also, HMA storage of barley at '35% moisture' without and with enzyme combination enhanced phytate degradation (43–58%) and P solubility (2.5–3.3 fold) ($P < 0.05$). Thus, HMA stored barley (solely endogenous enzymes) enhanced CTTAD of P to almost the same extent as dry stored barley with enzyme combination (endogenous enzymes plus added enzymes) in the barley diet (45–49%) and the barley-SBM diet (15–25%) ($P < 0.05$). Adding the enzyme combination to HMA stored barley achieved the greatest increase of CTTAD of P in the barley diet (70%) and the barley-SBM diet (36%) ($P < 0.05$). The inclusion of the enzyme combination to HMA stored barley tended to enhance the calculated CTTAD of P of SBM (29%) compared with no enzyme combination ($P = 0.06$) due to the lower endogenous enzymes activity in HMA stored barley. It is necessary to add the enzyme combination to enhance P digestibility of SBM when fed together with HMA stored barley. Overall, HMA storage with enzyme combination is a potential method to enhance P digestibility of barley in pigs to reduce the dietary need for inorganic P addition and P excretion to the environment.

Abbreviations: AA, amino acids; CIAD, coefficient of ileal apparent digestibility; CTTAD, coefficient of total tract apparent digestibility; CP, crude protein; DM, dry matter; HMA, high moisture airtight; N, nitrogen; NPN, non protein N; NSP, non-starch polysaccharides; SBM, soybean meal; SEM, standard error of mean

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1. Introduction

Environmental concern related to excessive P and nitrogen (N) excretion is a challenge to modern pig production. Cereals are major components in pig diets but the digestibility of P and N is often low and contributes to the high P and N excretions. Non-starch polysaccharides (NSP) and phytate tightly embed protein and bind e.g. P, which may hinder a high digestibility of cereal nutrients (Eeckhout and De Paepe, 1994; Fincher, 1975). Microbial phytase degrades phytate and enhances coefficient of total tract apparent digestibility (CTTAD) of P up to 0.65 (Blaabjerg et al., 2012; Poulsen et al., 2007; Rodehutscord et al., 1996) whereas reported effects on coefficient of ileal apparent digestibility (CAID) of N in pigs are inconsistent (Cowieson et al., 2017; Selle and Ravindran, 2008). High moisture airtight (HMA) storage of barley may be a promising approach to release P, protein, and amino acids (AA) for absorption because the moist conditions in HMA storage activate plant and microbial enzymes to degrade NSP and phytate while avoiding negative side effects caused by microbial activity. Thus, HMA storage enhances soluble P (Niven et al., 2007; Ton Nu et al., 2015) and phytate degradation in corn (Abrams et al., 1976) and soluble N in barley (Åman et al., 1990; Ton Nu et al., 2015). Adding a combination of phytase, xylanase, β-glucanase and protease during HMA storage (up to 30% moisture) increased nutrient solubility in rolled barley but apparently without reaching the maximum increase in P and N solubility (Ton Nu et al., 2015). Thus, we hypothesize that HMA storage of rolled barley above 30% moisture with the added enzyme combination enhances P and N solubility and consequently increases the CTTAD of P in HMA stored compared to dry stored barley when fed alone or together with soybean meal (SBM). The aim of this study was as follows: (1) to define the optimal moisture level for maximum P and N solubility in HMA stored rolled barley as affected by enzymes present in barley and added enzymes, and (2) to determine the effect of HMA stored barley and enzyme combination on CTTAD of P in pigs fed barley alone or together with SBM (without supplementations of AA, P, calcium (Ca)).

2. Materials and methods

The current study comprises two experiments: Experiment 1 (*in vitro*) studied the effect of storage (dry storage vs. HMA storage at 29%, 35% and 40% moisture) and enzyme combination on the solubility of P, N, and protein of barley; Experiment 2 (*in vivo*) studied the effect of storage (dry storage vs. HMA storage at 35% moisture) and enzyme combination on CTTAD of P in pigs fed a barley diet or a barley-SBM diet. As the present study aimed to compare the direct effect of storage and/or enzyme combination on the digestibility of P in the rolled barley diet or the barley-SBM diet, the measured CTTAD and not the coefficient of total tract true or standardized digestibility of P was applied (Adeola, 2001).

2.1. Preparation of barley for Exp. 1 and Exp. 2

The barley used in Exp. 1 and Exp. 2 originated from the same batch, which was ground by a roller mill (Skiold crushers KB 200, SKIOLD A/S, Sæby, Denmark) and divided into two parts for dry storage or HMA storage. Rolled barley and not the unbroken grain was used to ensure contact between substrate and enzymes to study the potential effect of storage without or with added enzymes. Before storage, the rolled barley was added without or with a combination of phytase (Natuphos 5000G, BASF SE, Ludwigshafen, Germany) at 1000 FTU/kg diet; xylanase (Danisco Xylanase 8000G, Danisco Animal Nutrition, Marlborough, United Kingdom) at 4000 U/kg diet; β-glucanase (ECONASE Barley P700, AB Vista, Marlborough Wiltshire, United Kingdom) at 17,500 BU/kg diet; and protease (RONOZYME ProAct CT, DSM Nutritional Products Ltd, Basel, Switzerland) at 15,000 PROT/kg diet. The storage procedure was based on the method of Ton Nu et al. (2015).

2.2. Experiment 1—in vitro study

Rolled barley without or with the enzyme combination was either stored in 10-L covered plastic buckets at ambient temperatures (dry storage) or in vacuum packed plastic bags (400 g per samples) providing airtight conditions for barley at moisture levels of 29%, 35% or 40% at 15 °C (HMA storage). The procedures of HMA storage and dry storage were described in details by Ton Nu et al. (2015). Three samples per treatment were collected before storage (d 0) and after 49 d of storage and then frozen immediately. Samples were freeze-dried and milled using a 1-mm screen prior to analysis of pH, ash, crude protein (CP), total P and Ca, soluble N, soluble protein, soluble P, phytate P, and enzyme activity (phytase, xylanase and β-glucanase).

2.3. Experiment 2—in vivo study

The experimental protocol was approved by the Danish Animal Experiments Inspectorate, The Danish Ministry of Justice (Copenhagen, Denmark).

Forty-eight female pigs (43 ± 2 kg) were assigned randomly to the barley diet or to the barley-SBM diet in a 2 × 2 factorial design consisting of storage (dry storage vs. HMA storage at 35% moisture) and enzyme combination (without vs. with). Pigs were housed in stainless steel metabolism crates (70 × 135 cm) for total collection of urine and feces and were fed twice daily (0800 h and 1400 h). Feed consumption and feed refusals were recorded daily. The diets were fed for 12 d, which included 5 d for adaptation and 7 d for total collection of urine and feces. On d 5, pigs were fitted with the urine bladder catheters for separate collection of urine and feces twice daily. Urine was collected in containers with 40 mL sulfuric acid (H_2SO_4; 30%) to diminish ammonia evaporation. Collected feces was pooled during the 7 d period. Samples of diets, feces and urine were analyzed for Ca and P. Dry matter was determined in

diets and feces.

Based on the results of Exp. 1, HMA storage at 35% moisture was chosen for Exp. 2. Rolled barley (400 kg per bag) without or with the enzyme combination was either stored in plastic bags (dry storage) or in vacuum packed plastic bags providing an airtight condition at 35% moisture (HMA storage). The procedure of HMA storage was as follows: the moisture content of rolled barley (13%) was adjusted to 35% by adding tap water. The enzyme combination was added to the experimental diets with added enzymes at the same time as water. The bags were equipped with a valve to fill up carbon dioxide regularly to guarantee airtight condition during storage. Samples in triplicate were taken before storage (d 0) and after 49 d of storage and immediately frozen for further analysis. Samples were processed as described in Exp. 1 prior to the analysis of pH, ash, total P and Ca, soluble P, phytate P, and enzyme activity (phytase, and β-glucanase).

Dry stored barley and HMA stored barley were ground by a hammer mill (3.5 mm screen size). Based on the Danish recommendation for all nutrients except CP, P and Ca (Tybirk et al., 2014), the experimental diets were prepared according to Table 3. No crystalline amino acids, mineral phosphate or Ca were added. The dry stored barley was divided into two parts and mixed without or with SBM (30%; Table 3). The HMA stored barley diets were divided into small portions for each meal per day and kept in the freezer. For HMA stored barley-SBM diets, SBM was mixed with HMA stored barley just before feeding each meal.

2.4. Sample analysis

Dry matter was determined by oven drying at 103 °C for 20 h. Total P was analyzed by the method of Stuffins (1967). The determination of Ca (by the atomic absorption spectrophotometric) and Na (by the flame photometric) was based on the method 975.03 (AOAC, 2000a) with some modifications: dry ashing was performed at 450 °C for 3 h in Step 1 and for 1 h in Step 2. Chlorine analysis was based on the method of LaCroix et al. (1970). Total N content in diets was analyzed by the Dumas method (Hansen, 1989). Phytate P analysis was based on the method of Haug and Lantzsch (1983). Phytase activity was analyzed as described by Engelen et al. (1994) whereas β-glucanase activity was determined according to ESC standard analytical SAM043-01 using Glycazyme tablets (60 mg) as a substrate (Enzyme Services & Consultancy (ESC) Ltd, Wales, UK). Xylanase activity analysis was based on the colorimetric method using Xylazyme AC 60 g as a substrate (Megazyme, Wicklow, Ireland).

For the determination of soluble P, soluble N, and soluble protein, samples (30 g) were mixed with demineralized water (70 g) by use of a magnetic stirrer at $18 \times g$ for 10 min, and pH was measured (Radiometer, Copenhagen, Denmark). The supernatant was extracted after being centrifuged at $2000 \times g$ and 4 °C for 10 min, and water soluble P and water soluble protein were measured by a Roche Cobass C 111 analyzer (Roche Diagnostics GmbH, Mannheim, Switzerland). Water soluble protein was measured based on the biuret assay that quantifies tripeptides and larger polypeptides or protein (not free amino acids and dipeptides). Soluble N in the supernatant and total N of feed in Exp. 1 and Exp. 2 were measured by use of the Kjeltec AUTO 2400 Analyzer System (FOSS, Hillerød. Denmark) based on the Kjeldahl method 978.02 (AOAC, 2000b). Soluble N is the amount of total N solubilized in water which includes protein and non protein N (NPN).

2.5. Calculations

Crude protein (CP), the solubility of P, N, and protein and the ratio between phytate P and total P (Phytate P:Total P) were calculated as follows:

$$\text{CP (g/kg DM)} = \text{total N (g/kg DM)} \times k$$

Where k is 5.45 for barley (Mariotti et al., 2008).

Nitrogen (N) solubility = soluble N (g/kg DM)/total N (g/kg DM)

Protein solubility = soluble protein (g/kg DM)/CP (g/kg DM)

P solubility = soluble P (g/kg DM)/total P (g/kg DM)

Phytate P:Total P = phytate P (g/kg DM)/total P (g/kg DM)

Coefficient of total tract apparent digestibility of P in SBM was calculated by the difference from the barley-SBM diets and the respective barley diets as following:

$$\text{CTTAD of P in SBM} = (P_{\text{barley-SBM}} \times \text{DigP}_{\text{barley-SBM}} - a \times P_{\text{barley}} \times \text{DigP}_{\text{barley}})/(b \times P_{\text{SBM}})$$

Where: $P_{\text{barley-SBM}}$ = total P of the barley-SBM diet (g/kg DM); $\text{DigP}_{\text{barley-SBM}}$ = the determined CTTAD of $P_{\text{barley-SBM}}$ diet; a = proportion of barley in the barley-SBM diet; P_{barley} = total P of the respective barley (g/kg DM); $\text{DigP}_{\text{barley}}$ = the determined CTTAD of P in the barley diet; b = proportion of SBM in the barley-SBM diet; P_{SBM} = total P of SBM (g/kg DM).

2.6. Statistical analysis

Data were analyzed by the SAS software version 9.3 (SAS Inst. Inc., Cary, NC, USA). Results were presented as least square means (LS means) and standard error of means (SEM). *P*-values were considered significant when $P < 0.05$ and tendencies were declared at $0.05 \leq P \leq 0.1$. The results of Exp. 1 were analyzed by the GLM procedure using a model including the main effect of storage (dry storage vs. HMA storage at 29%, 35% and 40% moisture) and enzyme combination and their interactions on the results of samples

Table 1
Effect of storage (dry storage vs. high moisture airtight (HMA) storage at 29%, 35%, and 40% moisture) and enzyme combination on phytate P, Phytate P:Total P, nutrient solubility, phytase activity, and pH of barley after 49 d of storage in Exp. 1.

	Treatment[1]					SEM	*P*-value[2]		
	Enzyme	Dry storage	HMA storage						
Item			29%	35%	40%		S	E	S x E
DM, g/kg diet	–	875	717	656	606	1.70			
	+	869	716	652	610				
Total P, g/kg DM	–	3.43	3.39	3.41	3.39	0.04	0.690	0.629	0.471
	+	3.36	3.43	3.42	3.36				
Phytate P (g/kg DM)	–	1.77^{a}	1.60^{ad}	1.23^{b}	0.71^{c}	0.04			< 0.001
	+	1.73^{a}	1.50^{d}	0.47^{e}	0.003^{f}				
Phytate P:Total P	–	0.52^{a}	0.47^{ad}	0.36^{b}	0.21^{c}	0.01			< 0.001
	+	0.52^{a}	0.44^{d}	0.14^{e}	0.001^{f}				
P solubility	–	0.25^{a}	0.34^{a}	0.56^{b}	0.81^{c}	0.02			< 0.001
	+	0.31^{a}	0.47^{b}	0.86^{c}	1.00^{d}				
CP, g/kg DM	–	97	99	101	100	0.47	< 0.001	0.689	0.746
	+	98	99	101	100				
Nitrogen solubility	–	0.21^{a}	0.21^{a}	0.41^{b}	0.57^{c}	0.01			0.015
	+	0.20^{a}	0.23^{a}	0.45^{b}	0.67^{d}				
Protein solubility	–	0.16^{a}	0.15^{a}	0.25^{b}	0.33^{c}	0.01			< 0.001
	+	0.16^{a}	0.16^{a}	0.27^{b}	0.40^{d}				
Phytase, FTU/kg DM	–	450^{a}	40^{b}	37^{b}	33^{b}	18			< 0.001
	+	1660^{c}	1080^{d}	1010^{d}	1140^{d}				
Xylanase, U/kg DM	–	< 100		< 100					
	+	2550		4120					
pH	–	5.73^{a}	5.96^{a}	5.78^{a}	4.86^{b}	0.05			< 0.001
	+	5.81^{a}	5.97^{a}	5.72^{a}	4.38^{c}				

^{a,b}Means within a row or a column without a common superscript differ ($P < 0.05$).

[1] Treatments: Rolled barley was dry stored (dry storage) or high moisture airtight (HMA) stored (HMA storage) without (−) or with (+) the combination of microbial phytase (BASF SE, Ludwigshafen, Germany), xylanase (Danisco Animal Nutrition, Marlborough, United Kingdom), β-glucanase (AB Vista, Marlborough, United Kingdom), and protease (DSM Nutritional Products Ltd, Basel, Switzerland) for 49 d.

[2] S = storage effect (dry storage vs. HMA storage 29% moisture vs. HMA storage 35% moisture vs. HMA storage 40% moisture), E = enzyme combination effect (without vs. with enzyme combination); S x E = the interaction between storage and enzyme combination.

collected at d 49. The same procedure was used in Exp. 2 on total P, P solubility, Phytate P:Total P, phytase activity, DM, and pH of the experimental diets. The MIXED procedure was used to investigate the main effect of storage, enzyme combination and their interaction on the digestibility and balance of P and Ca in pigs fed the barley diets. The same procedure was used to analyse the results of the barley-SBM diets as well as on the calculated CTTAD of P in SBM. Pigs were the experimental unit for all analysis. Treatment differences were separated by the PDIFF option of SAS software.

3. Results

3.1. Experiment 1—in vitro study

The endogenous phytase activity in barley remained as anticipated constant during the dry storage for 49 d (average 440 FTU/kg DM). The added phytase activity in barley at d 0 (1500 FTU/kg DM) and in dry stored barley at d 49 (1200 FTU/kg DM) was greater than planned (1000 FTU/kg barley). The analyzed enzyme activity in barley without or with the enzyme combination at d 0 was below 100 and 2500 U/kg DM for xylanase, respectively and 5900 and 20,000 U/kg DM for β-glucanase, respectively. Thus, the added xylanase (2400 U/kg DM) and β-glucanase activity (14,100 U/kg DM) in barley were lower than planned (4000 and 17,800 U/kg barley).

Before storage, DM of HMA stored barley at 29%, 35%, and 40% were 716, 659, and 610 g/kg diet, respectively, which remained unchanged after 49 d of storage ($P > 0.05$, Table 1). As expected, Phytate P:Total P in barley was the same before and after dry storage for 49 d without (0.50 and 0.52, respectively) and with (0.52 and 0.52, repectively) the enzyme combination. The interaction between storage and enzyme combination was observed on phytate degradation and nutrient solubility in barley ($P < 0.001$; Table 1). This was because HMA storage enhanced phytate degradation (31 and 60%, respectively), and the solubility of P (124 and 224%, respectively), N (95 and 171%, respectively) and protein (56 and 106%, respectively) in barley at 35% and 40% moisture compared with dry storage ($P < 0.001$), whereas phytate degradation and nutrient solubility were just slightly increased in HMA stored barley at 29% moisture ($P > 0.05$). Moreover, the enzyme combination enhanced P solubility in HMA stored barley (23–54%) depending on moisture level compared with no enzyme addition ($P < 0.05$), whereas no change was observed in dry stored barley ($P = 0.59$). Adding the enzyme combination also improved the solubility of N (17%) and protein (by 21%) in HMA stored barley at 40% moisture. The degrading effect of the enzyme combination on phytate degradation was only observed in barley stored at 35% and

Table 2
Effect of storage (dry storage vs. high moisture airtight (HMA) storage at 35% moisture for 49 d) and enzyme combination on P solubility, phytate degradation, enzyme activity, and pH of barley prepared for Exp. 2.

	Treatment[1]						
	Enzyme	Dry storage	HMA storage[2]	SEM	*P*-value[2]		
Item					S	E	S x E
DM, g/kg diet	–	861	666	0.5			
	+	861	667				
Total P, g/kg DM	–	3.38	3.31	0.06	0.778	0.266	0.410
	+	3.25	3.29				
P solubility	–	0.23	0.77	0.01	< 0.001	< 0.001	0.254
	+	0.35	0.87				
Phytate P, g/kg DM	–	1.64[a]	0.93[b]	0.02			< 0.001
	+	1.66[a]	0.69[c]				
Phytate P:Total P	–	0.49[a]	0.28[b]	0.01			0.006
	+	0.51[a]	0.21[c]				
Phytase activity, FTU/kg DM	–	420	320	140	< 0.05	< 0.001	0.103
	+	1600	980				
Xylanase, U/kg DM	–	< 100	< 100				
	+	1630	2550				
β-glucanase activity, BU/kg DM	–	4290	3120	3430	0.105	0.002	0.175
	+	24,500	13,140				
pH	–	5.78[a]	6.02[b]	0.02			< 0.001
	+	5.80[a]	6.14[c]				

[a,b]Means within a row or a column without a common superscript differ ($P < 0.05$).

[1] Treatments: Rolled barley was dry stored (dry storage) or high moisture airtight (HMA) stored (HMA storage) without (−) or with (+) the combination of microbial phytase (BASF SE, Ludwigshafen, Germany), xylanase (Danisco Animal Nutrition, Marlborough, United Kingdom), β-glucanase (AB Vista, Marlborough, United Kingdom), and protease (DSM Nutritional Products Ltd, Basel, Switzerland) for 49 d.

[2] *P*-value: S = storage effect (dry storage vs. HMA storage at 35% moisture), E = enzyme combination effect (without vs. with enzyme combination); S x E = the interaction between storage and enzyme combination.

40% moisture (by 62 and 100%, respectively, $P < 0.001$). The pH of HMA stored barley at 40% with the enzyme combination was slightly lower compared with no enzyme addition ($P < 0.001$), whereas pH was not different among the other treatments ($P > 0.05$). In general, phytase activity in HMA stored barley was lower than in dry stored barley ($P < 0.001$).

3.2. Experiment 2—in vivo study

The added phytase (1500 FTU/kg DM) and xylanase (3600 U/kg DM) activities found in barley at d 0 were close to the planned level. In contrast, the added β-glucanase activity in barley at d 0 (21,300 U/kg DM) was greater than expected.

Before storage, DM of HMA stored barley at 35% moisture was 656 and 654 g/kg diet without and with the enzyme combination, respectively, and remained almost constant during storage for 49 d. The added phytase (1270 FTU/kg DM) and added β-glucanase (17,300 U/kg DM) activities found in HMA stored barley at d 0 were lower than planned whereas the added xylanase activity (6000 U/kg DM) was greater than planned.

High moisture airtight storage increased the P solubility of barley by on average 0.53 compared with dry storage ($P < 0.001$; Table 2). The added enzyme combination increased the P solubility of barley by 13% compared with no enzyme addition ($P < 0.001$; Table 2). The interaction between storage and enzyme combination occurred because the added enzyme combination increased pH and decreased Phytate P:Total P of HMA stored barley compared with no enzyme addition ($P < 0.05$) whereas no effects were observed in dry stored barley ($P > 0.05$; Table 2). Regardless of the enzyme combination, HMA stored barley resulted in lower Phytate P:Total P and lower phytase activity compared with dry stored barley ($P < 0.001$).

Soybean meal in the current study had a greater content of total P (7 vs. 3 g/kg DM) and Ca (2.9 vs. 0.5 g/kg DM) than barley. Furthermore, SBM had a greater phytate P content (2.0 g/kg DM) than dry stored barley (1.6 g/kg DM) and HMA stored barley (0.9 g/kg DM). Phytase activity of SBM was below detection level (< 50 FTU/kg DM). The diet composition and analyzed content are given in Table 3. Averaged over the levels of enzyme addition, the barley-SBM diets had greater total P (4.5 vs. 3.3 g/kg DM) and Ca (1.2 vs. 0.5 g/kg DM) than the pure barley diets (Table 3). Phytate P of HMA stored barley diets was lower than dry stored barley diets whereas the phytase activity was slightly lower in the diets based on HMA stored barley.

3.2.1. Barley diet

For the barley diets, the intake of DM, P, and Ca was similar among dietary treatments ($P > 0.05$; Table 4). There was no effect of storage or enzyme combination on CTTAD of DM in pigs ($P > 0.05$; Table 4) whereas HMA storage increased CTTAD of P of barley by 14–45% (highest in dry stored barley due to dissimilar ('delayed') effects of the added enzymes) ($P < 0.001$). At the same time, P excretion in urine was increased in pigs fed HMA stored barley ($P = 0.01$) and by inclusion of enzymes (< 0.001). Across storage (dry

Table 3
Ingredients and analyzed chemical composition of experimental diets in Exp. 2.

	Dietary treatment[a]							
	Barley diet				Barley-SBM diet			
	Dry storage		HMA storage		Dry storage		HMA storage	
Item	−E	+E	−E	+E	−E	+	−E	+E
Ingredient, % as-fed								
Barley	99.65	99.65	99.73	99.73	69.49	69.49	74.05	73.98
Soybean meal					30.16	30.16	25.65	25.72
Sodium chloride	0.35	0.35	0.27	0.27	0.35	0.35	0.30	0.30
Analyzed chemical composition								
DM, %	87	87	67	68	88	88	74	73
CP[b], g/kg DM	114	116	118	115	251	238	248	247
Total Ca, g/kg DM	0.5	0.5	0.5	0.5	1.2	1.2	1.2	1.3
Total P, g/kg DM	3.3	3.3	3.3	3.3	4.6	4.4	4.4	4.4
Phytate P, g/kg DM	1.8	1.8	0.7	0.4	1.9	2.0	1.9	1.4
Na, g/kg DM	1.8	1.7	1.7	1.9	2.0	1.6	1.7	1.7
Cl, g/kg DM	3.5	3.3	3.4	3.5	3.5	3.0	3.1	3.2
Phytase activity, FTU/kg DM	400	1870	280	1050	280	1010	230	920

[a] Dietary treatments: Rolled barley was dry stored (dry storage) or high moisture airtight stored (HMA storage) without (−E) or with (+E) the combination of microbial phytase (BASF SE, Ludwigshafen, Germany), xylanase (Danisco Animal Nutrition, Marlborough, United Kingdom), β-glucanase (AB Vista, Marlborough, United Kingdom), and protease (DSM Nutritional Products Ltd, Basel, Switzerland) for 49 d, ground and then mixed without (barley diet) or with soybean meal (barley-SBM diet).
[b] CP was calculated as N (g/kg DM) × 6.25.

Table 4
Effect of storage (dry storage vs. high moisture airtight (HMA) storage at 35% moisture for 49 d) and enzyme combination (without vs. with) on DM intake, coefficient of total tract apparent digestibility (CTTAD) of DM and P, and the balance of P and Ca in barley diets in Exp. 2 (LS means ± SEM).

	Dietary treatment[1]							
	Dry stored barley		HMA stored barley			*P*-value[2]		
Item	−E	+E	−E	+E	SEM	S	E	S x E
DM intake, g/d	1162	1167	1172	1142	34			
CTTAD of DM	0.874	0.890	0.879	0.880	0.046	0.658	0.092	0.134
P balance, g/d								
Intake	3.79	3.91	3.91	3.77	0.11			
Feces	2.28	1.66	1.58	1.21	0.12	< 0.001	< 0.001	0.317
Urine	1.27	1.83	1.71	2.19	0.13	< 0.01	< 0.001	0.714
Net absorption	1.51[a]	2.24[b]	2.33[bc]	2.56[c]	0.09			0.011
Retention	0.25	0.41	0.62	0.37	0.15	0.249	0.761	0.152
CTTAD of P	0.400	0.578	0.596	0.679	0.232	< 0.001	< 0.001	0.061
Digestible P, g/kg DM	1.31[a]	1.93[b]	1.99[b]	2.24[b]	0.08			0.029
Ca balance, g/d								
Intake	0.57	0.61	0.62	0.59	0.02			
Feces	1.05	0.63	0.65	0.43	0.06	< 0.001	< 0.001	0.083
Urine	0.04	0.04	0.05	0.02	0.02	0.780	0.381	0.377
Net absorption	−0.48[a]	−0.02[b]	−0.03[b]	0.16[c]	0.05			0.009
Retention	−0.53[a]	−0.07[b]	−0.08[b]	0.13[c]	0.05			0.036
CTTAD of Ca	−0.849[a]	−0.291[b]	−0.508[b]	0.267[c]	0.806			0.003

[a,b]Means within a row or a column without a common superscript differ ($P < 0.05$).
[1] Dietary treatments: Rolled barley was dry stored or high moisture airtight (HMA) stored without (−E) or with (+E) the combination of microbial phytase (BASF SE, Ludwigshafen, Germany), xylanase (Danisco Animal Nutrition, Marlborough, United Kingdom), β-glucanase (AB Vista, Marlborough, United Kingdom), and protease (DSM Nutritional Products Ltd, Basel, Switzerland) for 49 d.
[2] *P*-value: S = storage effect (dry storage vs. HMA storage at 35% moisture), E = enzyme combination effect (without vs. with enzyme combination); S x E = the interaction between storage and enzyme combination.

or HMA), inclusion of the enzyme combination during the storage of barley resultated in a mean increase in CTTAD of P by 0.13 coupled with an increase in urinary P excretion by 0.52 g/d ($P < 0.001$; Table 4).

The interaction between storage and enzyme combination influenced P absorption ($P = 0.011$) and Ca retention ($P = 0.036$; Table 4). Without added enzymes, pigs fed the HMA stored barley showed greater P absorption (0.82 g/d) and derived Ca retention (0.45 g/d) compared with pigs fed dry stored barley ($P < 0.001$), whereas the effect of storage on P absorption and the resulting Ca

Table 5
Effect of storage (dry storage vs. high moisture airtight (HMA) storage at 35% moisture) and enzyme combination (without vs. with) on DM intake, coefficient of total tract apparent digestibility (CTTAD) of DM, P and Ca, and the balance of P and Ca in barley-soybean meal (SBM) diet in Exp. 2 (LS means ± SEM).

	Dietary treatment[a]							
	Dry stored barley-SBM		HMA stored barley-SBM			*P*-value[b]		
Item	−E	+E	−E	+E	SEM	S	E	S x E
DM intake, g/d	1223	1233	1177	1189	16	0.015	0.515	0.928
CTTAD of DM	0.869	0.872	0.875	0.881	0.050	0.136	0.380	0.776
P balance, g/d								
Intake	5.60	5.41	5.12	5.29	0.07			0.025
Feces	3.03	2.32	2.45	2.00	0.11	< 0.001	< 0.001	0.199
Urine	0.92	1.41	1.20	1.87	0.11	0.003	< 0.001	0.428
Net absorption	2.57	3.08	2.68	3.29	0.10	0.035	< 0.001	0.481
Retention	1.65	1.67	1.47	1.43	0.15	0.161	0.936	0.800
CTTAD of P	0.458	0.570	0.523	0.623	0.183	< 0.001	< 0.001	0.668
Digestible P, g/kg DM	2.10	2.50	2.28	2.77	0.08	0.003	< 0.001	0.718
Ca balance, g/d								
Intake	1.46	1.50	1.42	1.50	0.02	0.311	0.007	0.438
Feces	1.44	1.14	1.22	0.90	0.08	< 0.001	< 0.001	0.764
Urine	0.005	0.003	0.12	0.03	0.08	0.647	0.757	0.764
Absorption	0.02	0.36	0.20	0.60	0.09	< 0.001	< 0.001	0.479
Retention	0.17	0.38	0.14	0.53	0.16	0.646	0.364	0.328
CTTAD of Ca	0.106	0.244	0.137	0.403	0.588	< 0.001	< 0.001	0.556

[a] Dietary treatments: Rolled barley was dry stored or high moisture airtight (HMA) stored without (−E) or with (+E) the combination of microbial phytase (BASF SE, Ludwigshafen, Germany), xylanase (Danisco Animal Nutrition, Marlborough, United Kingdom), β-glucanase (AB Vista, Marlborough, United Kingdom), and protease (DSM Nutritional Products Ltd, Basel, Switzerland) for 49 d, ground and then mixed with soybean meal.

[b] *P*-value: S = storage effect (dry storage vs. HMA storage at 35% moisture), E = enzyme combination effect (without vs. with enzyme combination); S x E = the interaction between storage and enzyme combination.

retention (0.32 and 0.20 g/d) were smaller when the enzyme combination was added ($P < 0.05$; Table 4). Moreover, the effect of enzyme addition on P absorption was only significant in dry stored barley ($P < 0.001$) but not in HMA stored barley ($P = 0.287$). The content of digestible P was 0.68 g/kg DM greater in HMA stored barley without the enzyme combination ($P < 0.001$) and 0.31 g/kg DM with the enzyme combination ($P = 0.013$) compared to dry stored barley.

3.2.2. Barley-SBM diet

In barley-SBM diets, the DM intake of HMA stored barley were lower than of dry stored barley by 45 g/d ($P = 0.015$; Table 5) but the enclosure of DM intake as a covariate in the statistical model revealed no change in all parameters compared with the model without the covariate. With no enzyme addition, pigs fed the dry stored barley-SBM diet had a greater P intake by 0.48 g/d than pigs fed HMA stored barley-SBM diet ($P = 0.002$) and by 0.12 g/d when the enzyme combination was added (interaction between storage and enzyme combination, $P = 0.025$; Table 5). Ca intake was greater by an average of 0.06 g/d in pigs fed diets with the enzyme combination ($P = 0.007$; Table 5).

Inclusion of HMA stored barley increased the CTTAD of P of the barley-SBM diet on average 22% ($P < 0.001$) compared with dry stored barley (Table 5). However, the P excretion pathways changed so that the urinary P excretion was greater in pigs fed the HMA stored barley-SBM diet than in pigs fed the dry stored barley-SBM diet ($P = 0.003$) whereas the corresponding fecal excretion was lower. Consequently, the digestible P content was on average (with or without enzyme combination) 0.22 g/d greater in HMA stored compared with dry stored barley-SBM diets ($P = 0.003$; Table 5).

The addition of phytase, xylanase, beta-glucanase, and protease during storage increased CTTAD of P (by 0.11) and digestible P (by 0.45 g/kg DM) compared with no enzyme addition ($P < 0.001$, Table 5). However, no effect of storage or enzyme combination on the retention of Ca and P was observed ($P > 0.05$, Table 5). In general, the Ca intake was as planned very low (not balanced diets) and limited the overall retention of both P and Ca (Table 5).

The calculated CTTAD of P of SBM was based on the obtained results of barley (dry stored vs. HMA stored) without or with the enzyme combination by assuming additivity of CTTAD of P of the feedstuffs (Table 6). The CTTAD of P of SBM fed together with dry stored barley was calculated to be on average 0.07 greater compared with SBM fed together with HMA stored barley and almost identical without or with the enzyme combination ($P = 0.043$). Additionally, mixing SBM and HMA barley stored with the enzyme combination tended to increase CTTAD of P of SBM by 0.13 compared with no enzyme addition ($P = 0.06$).

4. Discussion

Difficulties in taking homogeneous samples from the mixture of rolled grains and added enzymes contribute to the deviation

Table 6
Coefficient of total tract apparent digestibility (CTTAD) of P in soybean meal (SBM) calculated from diets based on dry stored barley and high moisture airtight (HMA) stored barley without or with the enzyme combination.

Basal diet[1]	CTTAD of P in barley diet	CTTAD of P in barley-SBM diet	Calculated CTTAD of P in SBM	SEM	Mean	*P*-value[2]		
						S x E	S	E
Dry stored barley	0.400	0.458	0.578	0.419	0.581[a]	0.080	0.043	0.057
Dry stored barley + E	0.578	0.571	0.584					
HMA barley	0.596	0.523	0.446		0.510[b]			
HMA barley + E	0.769	0.623	0.574					

[1] Basal diets: Rolled barley was dry stored or high moisture airtight (HMA) stored without or with (+E) the combination of microbial phytase (BASF SE, Ludwigshafen, Germany), xylanase (Danisco Animal Nutrition, Marlborough, United Kingdom), β-glucanase (AB Vista, Marlborough, United Kingdom), and protease (DSM Nutritional Products Ltd, Basel, Switzerland) for 49 d, ground and then mixed without (barley-diet) or with soybean meal (barley-SBM).

[2] *P*-value: S = storage effect (dry storage vs. HMA storage at 35% moisture), E = enzyme combination effect (without vs. with enzyme combination); S x E = the interaction between storage and enzyme combination.

between the analyzed enzyme activity and the planned levels in both experiments.

4.1. Experiment 1—in vitro study

In Exp. 1, HMA storage of barley at 35% and 40% moisture enhanced the phytate degradation and the solubility of P, N, and protein compared with dry storage. The protein solubility of HMA stored barley at 35% and 40% moisture in the current study was similar to the observed results of barley soaked with acetic acid or lactic acid for 12 h (0.25–0.28) and 48 h (0.28–0.30), respectively (Christensen et al., 2014). The enhanced nutrient solubility of barley is most likely a result of endogenous enzyme activation, grain matrix degradation, and release of P caused by the moist conditions during HMA storage and maybe also microbial fermentation (Åman et al., 1990; Humer et al., 2013; Prigge et al., 1976). Microbial fermentation often results in the production of lactic acid, which is in line with the small decrease in pH of HMA stored cereals. Nevertheless, the magnitude of the effect of endogenous enzymes and microbial fermentation on the enhancement of the nutrient value of HMA stored cereal clearly depends on the level of endogenous enzyme activity and grain moisture content. Corn possesses a negligible phytase activity, so the hydrolysis of phytate in HMA stored corn was mainly due to lactic acid fermentation associated with a pH drop to 4 and a greater lactic acid concentration after storage (Humer et al., 2013). In the current study, a decrease in pH to below 5 was only observed in HMA stored barley at 40% moisture which indicates that fermentation to some extent may have occurred, whereas the observed pH (5.7–6) after storage at moisture levels up to 35% indicates that fermentation occurred to a very limited extent. The current findings imply that endogenous enzymes in barley were mainly responsible for enhancing nutrient solubility in barley at up to 35% moisture, whereas fermentation may play a role at moisture levels above 35% during HMA storage.

The current study showed a higher Phytate P:Total P and a lower nutrient solubility in HMA stored barley at 29% moisture compared with storage at 35% and 40% moisture. These results are in accordance with Baron et al. (1986) who reported that increasing moisture levels enhanced the N solubilization in maize. Ton Nu et al. (2015) also observed a positive relationship between moisture levels and N and P solubility as well as phytate degradation.

Carlson and Poulsen (2003) observed that soaking barley for 72 h at 20 °C led to a faster degradation rate of phytate compared with 10 °C. The same temperature depending effect was observed in the present study (15 °C) compared with the results obtained at a slightly greater HMA storage temperature at 15–18 °C (Ton Nu et al., 2015). Both studies used the same barley cultivar (*Zephyr*); however, the solubility of P (0.34) and N (0.21) in the current HMA stored barley at 29% was lower compared with that in the former study (0.38 and 0.30, respectively) (Ton Nu et al., 2015). Moreover, high moisture airtight storage of barley at 35% moisture enhanced phytate degradation and P solubility to a greater extent in Exp. 1 compared with Exp. 2. This is most likely due to the differences in storage scale (lab scale (400 g) vs. barn scale (400 kg)) and storage conditions (incubator with a controlled temperature at 15 °C vs. an averaged ambient temperature of 9 °C, respectively). The temperature fluctuations observed in the barn (Exp. 2) may affect and delay the activation or activity of enzymes and thus reduce the degradation rate of phytate. Moreover, another reason could be a large variation in phytate P content and lower inositol phosphate among barley samples which appeared more varied compared to other cereal grains

Adding the enzyme combination to HMA storage reduced Phytate P:Total P and increased P solubility in barley at both 35% and 40% moisture whereas the solubility of N and protein was solely increased in barley stored at 40% moisture. This is in agreement with the previous finding showing that the addition of exogenous enzymes to HMA stored barley was more effective in enhancing P than N solubility (Ton Nu et al., 2015).

Svihus et al. (1997) observed that HMA storage of barley at 40% moisture reduced protein (14%–32%) and AA contents (especially Arg and Lys) and increased ammonia loss (8%–13%) compared with dry storage. Increasing the grain moisture to an extreme level may result in microbial fermentation that promotes protein breakdown and increases the N solubility as well as the ammonia and amine contents within the soluble N fraction (Baron et al., 1986; Pieper et al., 2011; Svihus et al., 1997). Some concerns about using HMA stored barley in pig diets should be taken into account because an excessive increase in N solubility combined with

high fractions of ammonia and amines would be of no benefit to pigs. Also, a rapid decline in pH and a huge lactic acid production may not be favorable for the endogenous phytase activity and the phytate degradation as mentioned by Carlson and Poulsen, 2003. High moisture airtight stored triticale or wheat at 35% moisture accompanied by a high lactic acid production that decreased the pH to 3.9, consequently failed to improve CTTAD of P in pigs (Pieper et al., 2011). Thus, although HMA storage of barley at 40% moisture achieved the lowest (negligible) amounts of phytate and the greatest nutrient solubility in the current study, 35% moisture was chosen for Exp. 2 to prevent the latent loss of energy and N via ammonia due to microbial fermentation which was nil or very limited during HMA storage of barley at 35% moisture.

4.2. Experiment 2—in vivo study

Total P content in diets and digesta consists of water soluble inorganic P and other forms of P like soluble and insoluble organic phosphates bound to phytate, protein, or other large molecules (Ajakaiye et al., 2003). However, only P in the form of water soluble inorganic P is ready for absorption. Therefore, the increase in P solubility – indicating the release of P from phytate – was used as a rapid indicator for the enhancement of P availability of cereals in pigs in previous studies (Columbus et al., 2010; Niven et al., 2007). The current study also found that the enhancement in CTTAD of P of barley due to HMA storage and enzyme combination correlated positively with the increase in P solubility.

The current effect of HMA storage on enhancing CTTAD of P in barley was also observed in triticale and wheat at 25% moisture (0.32 and 0.22, respectively) (Pieper et al., 2011), and corn at 23% and 25% moisture (0.14 and 0.09) (Humer et al., 2013). Additionally, CIAD of P increased from 0.23 to 0.38 in HMA stored barley (29% moisture) compared with dry barley (Weltzien and Aherne, 1987). Likewise, Svihus et al. (1997) also found an increase of CIAD of P in broilers fed HMA stored barley, oat, or wheat at 40% moisture (0.19, 0.11, 0.09, respectively).

High moisture airtight storage enhanced CTTAD of P to a lower extent in the barley-SBM diet compared with the barley diet (6% vs. 15%, respectively). This was mainly due to a lower phytate P reduction (45%) caused by HMA storage in the mixed barley-SBM diet compared with the pure barley diet (67%). Similar trends were observed in HMA stored corn (29% moisture) where the CTTAD of P was enhanced by 0.08 in the mixed corn-barley-SBM diet (Humer et al., 2014) but 0.14 in the corn diet (Humer et al., 2013). These results clearly indicate that the enhancing effects of HMA storage of cereals did not result in further synergistic effects, when the cereals were mixed with other non-HMA stored feedstuffs.

Enzymes require moist conditions to be activated and sufficient time for the enzymes' active sites to access substrates. When directly supplied to dry stored barley, the shortage of time for phytate degradation in the anterior part of the gastrointestinal tract becomes the limiting factor that affects the efficacy of both endogenous enzymes in barley and added microbial enzymes to improve CTTAD of P. Thus, in the current study, the addition of the enzyme combination to dry stored barley (endogenous enzymes plus added enzymes) enhanced CTTAD of P to the same extent as HMA storage of barley at 35% moisture for 49 d (solely endogenous enzymes). Adding the enzyme combination to HMA stored barley at 35% moisture provided the endogenous enzymes in barley and the added microbial enzymes more time to degrade their substrates resulting in the greatest enhancement of CTTAD of P in pigs (70%).

In the current study, barley and SBM were the sole sources of dietary P and Ca, so the daily Ca intake (< 1 g/d) was extremely low and did not fulfill the pig's requirement, which resulted in the negative Ca absorption, retention, and digestibility. Calcium and P homeostasis, however, is tightly regulated (Blaabjerg et al., 2015). The low dietary Ca stimulates a rapid release of parathyroid hormone (PTH) (Brown, 1991) that consequently decreases renal P reabsorption and increases urinary P excretion (Klein, 2013). Thus, as long as the amounts of Ca and P for bone mineralization are imbalanced, increasing the digestible P of cereals will not result in enhanced P retention, and P excretion simply shifts from fecal to urinary elimination. As a consequence of the insufficient dietary Ca supply, the observed increase in CTTAD of P by HMA storage and enzyme combination just resulted in a greater urinary P excretion of HMA stored barley when fed alone (26%) or in combination with SBM (24%) compared with that of dry stored barley. This confirmed that the major site of regulating P homeostasis in pigs is the kidney and not the intestine. Létourneau-Montminy et al. (2012) also found that 37% of the digestible P was lost in the urine in pigs fed a diet with low dietary Ca (5 g/kg diet) compared with 9% loss in pigs fed a diet containing 8 g Ca/kg diet. Decreasing the dietary Ca content from 0.010 to 0.005 g/kg reduced the P retention by 8.3% and increased the P excretion in urine 13-fold (Narcy et al., 2010). Consequently, as long as the Ca requirement is not fulfilled, increasing dietary Ca will enhance the P retention and reduce the urinary P excretion. This was also seen in the current study, where an increase in Ca intake from 0.60 g/d in the barley diet to 1.47 g/d in the barley-SBM diet reduced the urinary P excretion from 1.75 to 1.35 g/d, respectively. Consequently, it is expected that the increase in P retention and the coinciding reduction in P excretion after HMA storage with enzyme addition will be greater if the Ca supply is raised. Thus, the Ca supplement to pig diets should at the same time maximize P digestibly and support maximum P retention. Poulsen et al. (2010) suggest that a dietary Ca content of around 6 g/kg would be sufficient to support a maximized P retention in growing-finishing pigs fed microbial phytase supplemented diets. Due to the coinciding Ca and P demand for mineralization, Ca absorption was increased together with the increase of digestible P (Blaabjerg et al., 2015, 2010). Therefore, these studies suggest to use the ratio between Ca and digestible P (Ca:dP) instead of Ca:P to formulate a proper dietary Ca supply in pig diets. The low energy concentration in barley diets and barley-SBM diets, which fulfilled 60% and 71% of energy requirement in pigs, respectively, was also expected to reduce the over-all daily retention of Ca and P in the current study due to a slower growth and bone development (McDowell, 2003).

Recent studies have confirmed that the addition of microbial phytase increased the CTTAD (and STTD) of P and Ca in diets but not in inorganic Ca supplements (González-Vega et al., 2015; Maison et al., 2015). Interestingly, Zhai and Adeola (2013) reported that there was an additive relationship between true total tract digestibility of P in corn and SBM in the corn-SBM diet. Fandrejewski et al.

(1997), however, concluded that the CTTAD of P of SBM is influenced by diet type, because SBM had a lower P digestibility when fed together with corn (0.24) compared with barley (0.28) or wheat (0.37). This is due to the high endogenous phytase activity in barley and wheat that also degrades phytate of SBM, whereas the negligible phytase activity in corn did not degrade phytate of SBM (Fandrejewski et al., 1997). Poulsen et al. (2019) also report that intrinsic barley/wheat phytases increased the CTTAD of P in corn when corn was mixed with wheat and barley. These results clearly demonstrate that additivity between feedstuffs can only be expected when no phytase is present. Interestingly, the calculated CTTAD of P of SBM in the current study was quite high (0.45–0.58) compared with the results of Fandrejewski et al. (1997) (0.24–0.37) and Zhai and Adeola (2013) (0.36–0.38). This difference may be due to e.g. the dissimilarity in the SBM grade or cultivar and soybean processing. The calculated CTTAD of P of SBM based on the dry stored barley diet was greater compared with that of the HMA stored barley diet. This is most likely because of the lower phytase activity in the HMA stored barley than in the dry stored barley at feeding. This also agrees with previous studies that observed a decrease in endogenous phytase during incubation or soaking of cereals (Carlson and Poulsen, 2003; Christensen et al., 2014). Moreover, addition of the enzyme combination to the barley had no effect on the calculated CTTAD of P of SBM in dry stored barley. Thus, barley seemed to be the bottleneck for improvements in CTTAD of P in pig diets, because the endogenous phytase in barley could degrade phytate and improve the CTTAD of P of SBM to the same extent as with addition of the enzyme combination. The numerical lower calculated CTTAD of P of SBM (0.13) in HMA stored barley diet with no enzyme addition compared with that added with the enzyme combination could be explained by the decrease of endogenous enzymes in barley after HMA storage. Consequently, it is necessary to add microbial enzymes to enhance the calculated CTTAD of P of SBM when fed together with HMA stored barley to compensate for the decrease in endogenous barley enzymes activity during storage. To break the barrier for further improvements in cereal P digestibility, detailed studies on how to break down the seed structures involving physical and enzymatic processes are required.

5. Conclusion

Overall, 35% moisture seems to be the optimum moisture level for HMA storage of rolled barley without or with enzyme combination to enhance phytate degradation and the solubility of P, N and protein and at the same time prevent the latent loss of energy and N via ammonia during HMA storage. The enhancement in CTTAD of P of barley reveals the potential of HMA storage with enzyme combination as means of reducing the need for dietary inorganic P supplement and lessen P excretion to the environment. The barrier for further improvements in cereal P digestibility requires optimization of the processes leading to breakdown of cell wall structures.

Declaration of Competing Interest

The authors have no conflicts of interests in this work.

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