Environmental concerns due to the high excretion of phosphorus (P) and nitrogen (N) via manure-amended per hectare are a challenge to the highly intensive pig production. This is mainly due to the low digestibility of P and N in the cereal part and aimed to provide knowledge on using high-moisture (90% dry matter) storage of barley with exogenous enzymes on P and protein in barley during storage and the solubilisation of P and amino acids in pigs.
HIGH MOISTURE AIRTIGHT STORAGE OF BARLEY WITH EXOGENOUS ENZYMES: AN APPROACH TO INCREASE THE DIGESTIBILITY OF PHOSPHORUS, PROTEIN AND AMINO ACIDS IN PIGS?

MAI ANH TON NU
PhD THESIS – SCIENCE AND TECHNOLOGY – 2015

AARHUS UNIVERSITY
High moisture airtight storage of barley with exogenous enzymes:  
An approach to increase the digestibility of phosphorus, protein and amino acids in pigs?

MAI ANH TON NU

PhD THESIS – DEPARTMENT OF ANIMAL SCIENCE – 2015
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Dedicates to Mom and Dad: Finally, I understand your feelings when you did your PhD.

Thank you for encouraging me to explore the world and do what I love.
Preface

This PhD is submitted to the Graduate School of Science and Technology, Aarhus University to meet the requirements for the degree of Doctor of Philosophy (PhD). The PhD study was conducted from June 2012 to June 2015 in Animal Nutrition and Environmental Impacts Group at Foulum Research Centre of Aarhus University. The project was granted by The Ministry of Food, Agriculture and Fisheries of Denmark, The Danish AgriFish Agency and Aarhus University. The principal supervisor of the PhD study is Section Manager – Professor Hanne Damgaard Poulsen and the co-supervisor is Assistant Professor Karoline Blaabjerg.

The overall aim of the PhD study was to provide an improved knowledge on the effects of high moisture airtight (HMA) storage with exogenous enzymes on the solubilisation of phosphorus, nitrogen and protein of cereals during storage in relationship with the digestion and absorption of phosphorus, protein and amino acids in the digestive tract of pigs. The enhancement in phosphorus, protein and amino acids digestibility of barley by HMA storage with exogenous enzymes will reduce the need for dietary protein feedstuffs and inorganic phosphorus supplement and decrease the excretion of phosphorus and nitrogen to environment.
Acknowledgements

I would like to express my deep gratitude to my main supervisor, Professor Hanne Damgaard Poulsen, who has nurtured my science side from a Master’s student to a PhD fellow, and her knowledge and passion for science have always inspired me to keep exploring and learning. I would like to thank my co-supervisor, Assistant Professor Karoline Blaabjerg for her guidance and support, and her suggestions and ideas have always broadened my point of view. I am deeply grateful to my supervisors for their patience and great efforts on supporting me to complete the manuscripts and this thesis.

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I would also like to thank all my fellow PhD and Master students for their supports, especially Charlotte Gaillard – the best officemat and housemate ever; Elham Assadi Soumeh – the best thesis-writing partner and a super model; Jesper Bjerg Christensen – the
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Mai Anh Ton Nu
Denmark, June 22nd, 2015.
Summary

Environmental concerns due to the high excretion of phosphorus (P) and nitrogen (N) via manure are a challenge to modern pig production. This is mainly due to the low digestibility of N and P and the amino acid profile of the cereal part that often constitute more than 70% of a typical North European pig diet. Cereal P and protein are poorly digested by pigs, because non-starch polysaccharides (NSP) and phytate tightly embed protein and amino acids and bind P in the grain matrix. The endogenous enzymes in cereals and the added microbial enzymes can degrade NSP and phytate; however, the short retention time and unfavourable conditions for NSP and phytate degradation in the digestive tract limit the efficacy of exogenous enzymes on enhancing the digestibility of P and N of cereals in pigs. In this context, high moisture airtight (HMA) storage with exogenous enzymes may be an effective approach to enhance the digestibility of P, N and amino acids of cereals in pigs.

Focus of this PhD study was the cereal part. The PhD study aimed to provide an improved knowledge on the effect of HMA storage with a combination of phytase, xylanase, β-glucanase and protease on the phytate degradation and the solubilisation of P, N and protein of barley and triticale during storage and subsequently on the digestion and absorption of P, N and amino acids of barley in the digestive tract of pigs. It is hypothesised that HMA storage of cereals creates moist conditions that activate the endogenous cereal enzymes and the enzyme combination and provide more time for the enzymes to degrade NSP and phytate in order to release P and N before feeding. As a result, this will increase the digestibility of P, N and amino acids of cereals in pigs.

This PhD study is composed of 3 experiments. Experiment 1 evaluated the effect of grain moisture (20%, 23%, 26% and 29% moisture), grain processing (whole vs. rolled), storage time (0, 14, 29 and 49 days) and enzyme combination (without vs. with) on the phytate degradation and the solubility of P and N in HMA stored barley and triticale. Rolling grains with a grain moisture at a minimum of 26% is required before HMA storage of barley and triticale to enhance the phytate degradation and the solubility of P and N.

Experiment 2 evaluated the effect of storage of rolled barley (dry storage vs. HMA storage at 29%, 35% and 40% for 49 days) and the enzyme combination (without vs. with) to define the optimum moisture level for the maximum P, N and protein solubility in HMA.
stored barley. The 35% moisture level seems to be the optimum for HMA storage of rolled barley to enhance the phytate degradation and the solubility of P, N and protein and to prevent the loss of energy and N (via ammonia), as fermentation was nil or very limited under this condition.

In Experiments 1 and 2, the inclusion of the enzyme combination to rolled barley (29%, 35% and 40% moisture) and rolled triticale (26% and 29% moisture) during HMA storage enhanced the phytate degradation and the solubility of P and N to a greater extent compared with no enzyme addition (P<0.05).

Experiment 3 evaluated the effect of storage of rolled barley (dry storage vs. HMA storage at 35% for 49 days) without or with the enzyme combination on the apparent total tract digestibility (ATTD), the balance of P and N and the apparent ileal digestibility (AID) of protein and amino acids when fed to pigs alone (barley diet) or in combination with soybean meal (SBM) (barley-SBM diet). HMA stored barley with the enzyme combination resulted in the greatest increase in ATTD of P in the barley diets (70%) and in the barley-SBM diets (36%) (P<0.05). Nevertheless, a shift of P excretion from faeces to urine was observed in pigs fed HMA stored barley with the enzyme combination alone or together with SBM, because the dietary Ca was insufficient to support an increase in P retention (P<0.05). HMA storage with the enzyme combination also enhanced the AID of protein (16%), the AID of Lys, Met and Arg (10-15%) and the digestible amount of Lys, Met+Cys, Ile and Phe (7-17%) compared with the dry storage in barley diets (P<0.05). However, there was no effect of HMA stored barley with the enzyme combination on the overall ATTD and AID of protein and the AID of amino acids when fed to pigs together with SBM (P>0.05). HMA storage with the enzyme combination also did not reduce the excretion of N to manure in pigs fed barley and barley-SBM diets. Moreover, the results indicate that the P and protein solubility of barley can be used as indicators to predict the digestibility of P and N of barley in pigs.

In conclusion, the enhancement of the ATTD of P and the AID of protein and indispensable amino acids of barley by HMA storage with the enzyme combination revealed a possibility to reduce the dietary need for inorganic P addition and protein feedstuffs and to decrease the excretion of P and N.
Resume


PhD.-afhandlingen omfatter 3 forsøg. Forsøg 1 vurderede effekten af kernefugtighed (20, 23, 26 og 29 % fugtighed), kerneforarbejdning (hel vs. knækket), opbevaringstid (0, 14, 29 og 49 dage) og enzymkombination (uden vs. med) på fytatnedbrydning og opløselighed af P og N i gastæt opbevaret byg og tritikale. Resultaterne viser, at forøget fytatnedbrydning og opløselighed af P og N forudsætter, at kernerne knæckkes før lagring, og at de skal lagres ved en fugtighed på minimum 26 % ifm. gastæt opbevaring.

Forsøg 2 vurderede effekten af opbevaring af knækket byg (tør opbevaring vs. gastæt opbevaring ved 29, 35 og 40 % i 49 dage) og enzymkombination (uden vs. med) for at finde det optimale fugtighedsniveau for maksimal P-, N- og protein-opløselighed i gastæt opbevaret byg. 35 % fugtighed var tilsyneladende det optimale fugtighedsniveau for gastæt opbevaring af knækket byg for at forøge fytatnedbrydeligheden og opløseligheden af P, N og protein og for at forhindre tab af energi og N (via dannelse af ammoniak), da fermenteringen var 0 eller meget begrænset under disse forhold.
I forsøg 1 og 2 blev fytatnedbrydeligheden og opløseligheden af P og N i højere grad forøget ved tilsætning af enzymkombinationen til knækket byg (29, 35 og 40 % fugtighed) og knækket tritikale (26 og 29 % fugtighed) under gastæt opbevaring.

Forsøg 3 vurderede effekten af opbevaring af knækket byg (tør opbevaring vs. gastæt opbevaring ved 35 % i 49 dage) uden eller med enzymkombinationen på den tilsyneladende fækal fordøjelighed (FK) og den tilsyneladende ileale fordøjelighed (IFK) af protein og aminosyrer, når det blev givet til svin alene (bygdicæt) eller i kombination med sojaskrå (byg-sojaskrå-dicæt). Gastæt opbevaret byg med enzymkombinationen resulterede i den største forøgelse af fordøjeligheden af P i bygdicæten (70 %) og i byg-sojaskrå-dicæten (36 %) (P<0.05). Et skift i udskillelsen af P fra fæces til urin blev observeret hos alle grise, der blev fodret med gastæt opbevaret byg med enzymkombinationen alene eller med sojaskrå, fordi Ca-forsyningen ikke var tilstrækkelig til at understøtte en forøget P-afløjring (P<0.05). Sammenlignet med tør opbevaring forøgede gastæt opbevaring med enzymkombinationen også IFK af protein (16 %), IFK af Lys, Met og Arg (10-15 %) og indholdet af fordøjeligt Lys, Met+Cys, Ile og Phe (7-17 %) i bygdicæterne (P<0.05). Der var dog ingen effekt af gastæt opbevaret byg med enzymkombinationen på den overordnede FK og IFK af protein og IFK af aminosyrer, når det gastætopbevarede byg blev givet sammen med sojaskrå (P>0.05). Gastæt opbevaring med enzymkombinationen reducerede i det aktuelle forsøg ikke udskillelsen af N fra svin, der blev fodret med byg- og byg-sojaskrådicæterne. De opnåede resultater indikerer sammenlagt, at P- og proteinopløseligheden af byg kan bruges som indikatorer til at prædiktere fordøjeligheden af P og N hos svin.

Konklusionen er, at forøgelsen i den tilsyneladende fækal fordøjelighed af P og den tilsyneladende ileale fordøjelighed af protein og essentielle aminosyrer i byg via gastæt opbevaring med enzymkombinationen giver mulighed for at reducere behovet for tilsætning af uorganisk P og proteinfoder (sojaskrå mv.) ved optimeret fodersammensætning. Sammenlagt vil dette give mulighed for at nedsætte udskillelsen af P og N i den moderne svineproduktion.
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   - **Phosphorus**

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List of Abbreviations

**ATTD**: Apparent total tract digestibility

**AID**: Apparent ileal digestibility

**CP**: Crude protein

**DM**: Dry matter

**HMA**: High moisture airtight

**N**: Nitrogen

**P**: Phosphorus

**SBM**: Soybean meal
Chapter I. Introduction

Over the last few decades, the modern industrialised pig production has increased rapidly and shifted to the highly intensive production. In Denmark, the number of pigs\(^1\) per hectare of agricultural land\(^2\) increased from 2.25 in 1961 to 4.81 in 2011 (FAOSTAT, 2015). The driving force behind this increase is a high demand for animal products along with the expansion of the global population predicted to reach 9.6 billion in 2050 (United Nations, 2013). The limited arable land and natural resources (e.g. phosphorus (P)) can hardly cope with the demand of cereals and protein crops for foods and feeds in the future. Moreover, the intensive pig production is associated with various environmental issues such as the high P and nitrogen (N) content excretion in manure amended per ha resulting in nitrate leaching, accelerated eutrophication of surface waters and ammonia emission (Fernández et al., 1999; Steinfeld et al., 2006).

The high excretion of P and N in manure is certain to arise from the pig diets which are often based on cereals supplemented with protein feedstuffs, e.g. soybean meal or rapeseed meal. Figure 1 presents an example of a very simple pig diet with 78% barley and 18% soybean meal for growing-finishing pigs. Although cereals are normally considered the main energy source, barley contributes 49% of dietary P and 47% of dietary protein due to its high proportion (above 70%) in pig diets (Figure 1). However, the utilisation of P and N is low (45% and 47%, respectively) in growing-finishing pigs leaving 55% of P intake and 53% of N intake excreted to manure (Figure 1). Figure 1 also reveals that the low N utilisation in pigs is associated with (1) a high urinary N excretion contributing to a high ammonia emission and (2) a low apparent ileal digestibility (AID) of protein of barley (73%) compared with that of soybean meal (SBM, 88%). However, the major problem in P utilisation is a low apparent total tract digestibility (ATTD) of P (a low amount of digestible P that is available for absorption) in both cereals (43% in barley) and SBM (39%) (Figure 1).

\(^{1}\) The total number of pigs is the number of pigs enumerated in Denmark anytime from October 1\(^{st}\) 1960 (or 2010) to September 30\(^{th}\) 1961 (or 2011)/total agricultural land (ha).

\(^{2}\) Agricultural land is the sum of areas under a) arable land (land under temporary agricultural crops, temporary meadows for mowing or pasture, land under market and kitchen gardens and land temporarily fallow. It is not meant to indicate the amount of land that is potentially cultivable); b) permanent crops and c) permanent meadows and pastures. FAO. 2014. FAO statistical yearbook 2014, Europe and Central Asia food and agriculture. p 113. FAO, Budapest, Hungary.
(A) Nutrient contribution from each ingredient in a barley-SBM diet

<table>
<thead>
<tr>
<th>Nutrient content (% as-fed basis)</th>
<th>Barley</th>
<th>Soybean meal (SBM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>8.7</td>
<td>42.7</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.28</td>
<td>0.66</td>
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<th>Digestibility, %&lt;sup&gt;1&lt;/sup&gt;</th>
<th>AID of protein</th>
<th>ATTD of phosphorus</th>
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| Diet composition, %                  | 78             | 18                |

(B) Utilisation of P and N in a growing pig (32-107 kg) fed barley-SBM diets<sup>2</sup>

Figures 1. Phosphorus (P) and nitrogen (N) contribution from each ingredient (A) and the utilisation of P and N (B) in a simple cereal-soybean meal diet for growing finishing pigs (32-107 kg).

Diet composition: 78% barley and 18% soybean meal – the dietary crude protein level is 14.5% and the dietary phosphorus level is 0.45%.

<sup>1</sup>Nutrient content, apparent ileal digestibility (AID) and apparent total tract digestibility (ATTD) of N of barley and SBM were according to (VSP, 2015). Monocalcium phosphate (Ca(H₂PO₄)₂ (22.9 % P as-fed basis, and ATTD of P, 67%) was used as inorganic P supplementation in the diet (VSP, 2015). Calculation of P and N utilisation was based on the Danish standards 2015 for pig production in Denmark (Poulsen, 2014).
The low digestibility of P and N of cereals in pigs is caused by:

(1) A high content of non-starch polysaccharides (NSP) and phytate that embed protein and bind P tightly in the grain matrix and hinder a higher digestibility of P, N and amino acids of cereals.

(2) A high amount of dispensable amino acids but a limited amount of indispensable amino acids.

To degrade the complex structure of NSP and phytate requires a combination of various enzymes which are not secreted or secreted only in negligible amounts along the pig’s digestive tract. Although the endogenous enzymes in cereals and the microbial enzymes can degrade NSP and phytate, these enzymes require moist conditions to be activated and a sufficient time to degrade the substrates. Moreover, the exogenous enzymes encounter many challenges, e.g. the difference in pH between the stomach and the small intestine, the short retention time and the proteolytic activities of digestive enzymes along the digestive tract. In this context, high moisture airtight (HMA) storage with exogenous enzymes may be a promising approach to enhance the P and N digestibility of cereals. HMA storage is a process of storing cereal grains at a high moisture content in airtight condition. Thus, the moist condition may activate the endogenous enzymes in cereals and the added microbial enzymes during storage to degrade NSP and phytate and to release P, protein and amino acids before feeding. In other words, cereals are “pre-digested” during storage, so the cereal P and protein become more digestible before feeding.

Overall, the modern pig production is under pressure from the increased feed price due to the limited crop supply and natural resources and the environmental concerns. Therefore, an enhancement in the P and N digestibility in pigs is desirable. For that reason, the current PhD study focuses on the cereal part and the effects of HMA storage with exogenous enzymes on the phytate degradation and the solubilisation of P, N and protein of barley and triticale during storage and then the digestion and absorption of P and N in the digestive tract of pigs. The results of this PhD study will pave a way to improve the P and N digestibility of cereals in pig. This will alleviate the environmental load, reduce the need for dietary protein feedstuffs and inorganic P supplement and, in the long run, resolve the shortage of natural resources.
Chapter II. **Background**

The aim of this chapter is to provide a knowledge base about the limiting factors that hinder the enhancement of P and N digestibility in pigs and the potentials to overcome them. Several aspects of P and N nutrition are explored at 3 different angles:

1. The digestion, absorption and excretion of P and N in pigs
2. The potential and drawback of cereals regarding P and N
3. Effect of various feed processing on enhancing digestibility of P and N in pigs.

### 1. Nutrient digestion, absorption and excretion in pigs

#### 1.1. **Nitrogen**

![Diagram of Nitrogen Digestion](image)

**Figure 2. The digestion and absorption of protein (Ball et al., 2011)**

The digestion and absorption of protein in the digestive tract is summarized in Figure 2. Hydrochloric acid (HCl) and pepsin initiate protein digestion in the stomach (Krehbiel and Matthews, 2003). Pepsin actively breaks the peptide bonds between Phe, Tyr, Leu, Val and Glu and other amino acids (Ulshen, 1987) and consequently degrades proteins into many large polypeptides (Krehbiel and Matthews, 2003). In the small intestine, pancreatic enzymes (trypsin, chymotrypsin and elastase) continue to degrade these polypeptides...
further into many smaller peptides and amino acids (Krehbiel and Matthews, 2003). The final products of this process are about 60% oligopeptides (up to 6 amino acids residues) and 40% of free amino acids (Alpers, 1994). Then, various peptidases in the microvillus membrane of the small intestine split these oligopeptides into tri-, dipeptides, and amino acids (Krehbiel and Matthews, 2003). During absorption, several transporters may be involved in carrying the intact tri- and dipeptides (the single H⁺-coupled transporters) and the free amino acids (the Na⁺-dependent and –independent transporters) (Krehbiel and Matthews, 2003). The major site of amino acid absorption is in the upper part of the small intestine. However, the age of the pigs and the diet composition may influence the absorption area to a certain extent (Buraczewska, 1981; Leibholz, 1989). Additionally, the absorption rate of different amino acids may vary depending on their concentration in the small intestine (Buraczewska, 1981). In the large intestine, the microorganisms degrade the N compounds from undigested digesta and the endogenous loss, and the end products of this process are ammonia, amines, volatile fatty acids and microbial amino acids (Yen, 2001). However, a negligible amount of amino acids is absorbed in the large intestine, and the faecal N consists partly of the undigested dietary protein but mainly of the intestinal bacteria and the endogenous N losses (Bender, 2007).

Figure 3. The Barrel Theory of amino acids, adapted from Ajinomoto (2015)

Pigs do not require protein per se but its constituent amino acids – especially the indispensable amino acids, which are the building blocks for protein synthesis in the body (Poulsen et al., 2013). An inadequate amount of 1 indispensable amino acid will prevent
the protein synthesis and leads to the deamination of other amino acids to ammonia that is excreted as urea via urine (Bender, 2007). This is illustrated by the Barrel Theory of amino acids (Figure 3). However, the excessive amounts of amino acids, especially dispensable amino acids, also result in their deamination and the increase in urinary N excretion which again may lead to ammonia emission. Therefore, a high urinary N excretion reflects (1) a surplus of dietary protein, (2) a limit of dietary energy on protein deposition and (3) an imbalance of amino acid profile compared with the pigs’ requirement (Aarnink and Verstegen, 2007; Gatel and Grosjean, 1992). Overall, the balance between the dietary amino acid supply and the pigs’ requirement is important from both a nutritional and an environmental point of view.

Moreover, there are several factors that influence the utilization of N and amino acids in pigs:

(1) Amino acids profile of feedstuffs
(2) Dietary energy supply
(3) Anti-nutritional factors, e.g. dietary fibre and phytate
(4) Feed processing
(5) Other factors, e.g. age and breed of pigs.

In fact, the digestion and absorption of protein and amino acids in pigs become more problematic when protein and amino acids of feedstuffs are bound by NSP and phytate and thereby are inaccessible for digestive enzymes. Unfortunately, monogastric animals like pigs are not capable of digest NSP and phytate very well. NSP and phytate are discussed in details in the next sections.

1.2. Phosphorus

In pigs, P is absorbed mainly in inorganic form as phosphate (PO₄³⁻), hydrogen phosphate (HPO₄²⁻) and di-hydrogen phosphate (H₂PO₄⁻) (Anderson, 1991). The inorganic phosphates come either directly from the dietary inorganic sources or indirectly from the organic sources after enzymatic hydrolysis (Jongbloed, 1987). P is absorbed at different rates and quantities along the small intestine (Breves et al., 2007). However, the major absorption of P occurs in the posterior part of the mid-jejunum (the proximal half of the small intestine), and is less efficient in the ileum (Partridge, 1978). Moreover, the jejunum actively absorbs P at a higher rate than the duodenum (Crenshaw, 2000). The stomach and the large intestine absorb a negligible amount of P compared with the small intestine (Partridge, 1978). Calcitriol (CT) simulates the P absorption predominantly in the small
intestine, together with other factors such as dietary P content, plasma pH and several hormones (estrogen and glucocorticoids) (Breves et al., 2007; Stauber et al., 2005).

Up to 80% of P in feedstuffs (cereals and seeds) is tightly bound in phytate (Poulson, 2000). However, pigs generate insufficient amounts of the phytate-degrading enzymes (phytase) to liberate the bound P from the phytate complex (Selle and Ravindran, 2008). Moreover, mucosal phytase and phosphatase are secreted mainly in the jejunum, and these enzymes have the highest efficiency on the lower esters of phytate (IP3) but the least efficiency on phytate (IP6) (Hu et al., 1996; Pointillart et al., 1985). The bottleneck of phytate degradation is to degrade phytate (IP6) to its lower ester (IP5), because the lower esters are degraded at a faster rate (Blaabjerg et al., 2011). The endogenous phytase from feedstuffs like cereals and the added microbial phytase are capable of degrading phytate. These exogenous phytase are activated in the acidic conditions of the stomach (Kemme et al., 2006; Yi and Kornegay, 1996). Also, the activities of endogenous phytase in cereals and the added microbial phytase are higher in the stomach than in the upper part of the small intestine, and are much lower in the lower part of the small intestine (Kemme et al., 2006; Rapp et al., 2001a; Yi and Kornegay, 1996). The study of Kemme et al. (2006) has also shown that 52% of phytate was degraded in the stomach, whereas only 13% was degraded in the small intestine. Consequently, these results indicate that the stomach is the major site for phytate degradation of the endogenous phytase in cereals and the added microbial phytase (Rapp et al., 2001b; Svihus, 2011). Also, because P is absorbed mainly in the proximal half of the small intestine, the digesta passage rate and retention time (especially in the stomach) turn out to be important because they influence the extent of phytate hydrolysis. Approximately 40% of the total P intake left the stomach within the first hour after feeding and 85% within 6 hours after feeding (Rapp et al., 2001b). Rapp et al. (2001a) also estimated that 60% of the microbial phytase and 40% of the endogenous phytase in feedstuffs left the stomach within the first 6 hours after feeding. In fact, the gastric phytate (IP6) degradation rate was highest within the first hour after feeding (Blaabjerg et al., 2011; Rapp et al., 2001b). However, about 30 to 45% of intact phytate (IP6) still passed on to the small intestine within the first 5 hours after feeding even after the addition of microbial phytases (Blaabjerg et al., 2011). Overall, the average retention time in the pig’s stomach is 2.5-6.0 hours and between 4 and 10 hours in the small intestine (Svihus, 2011). It is therefore a challenge for exogenous phytases to degrade phytate and liberate P before the absorption site. Moreover, the variation in pH (a low pH in the
stomach (average between 3.0 to 5.0) and a high pH in the small intestine (between 6.0 and 7.5)) and the proteolytic activities of digestive enzymes along the digestive tracts may reduce or even inhibit the activity of the endogenous enzymes in feedstuffs and the added microbial enzymes. Consequently, some studies have shown that the ATTD of P rarely exceeds 60 to 70% even with a high dose of microbial phytase supplied in cereal based diets (Kim et al., 2008; Poulsen et al., 2007; Weremko et al., 1997). Also, the ATTD of P of feedstuffs can vary in a broad range from 10 to 60% in pigs depending on their phytate content and the activity of endogenous phytase (Crenshaw, 2000). Consequently, the pig diets have been supplemented with inorganic phosphate to fulfil the P requirement, but the true P digestibility in different inorganic phosphate sources also varied between 54 to 67% (Poulsen, 2007). Thus, the P utilisation is still quite low in pigs (about 60%) even with the supplementation of microbial phytase (Poulsen et al., 2013).

The microorganisms in the large intestine are capable of secreting phytase to hydrolyse phytate effectively (Sandberg et al., 1993; Schlemmer et al., 2001). Nevertheless, because of the negligible amount of absorbed P in the large intestine, their activities do not contribute significantly to P utilisation and excretion in pigs (Moore and Tyler, 1955).

Overall, the pigs do not secrete or secrete insufficient amounts of enzymes to degrade NSP and phytate that encapsulate P, protein and amino acids in feedstuffs. The endogenous enzymes in feedstuffs and the added microbial enzymes are capable of degrading NSP and phytate. However, the short retention time and the unfavourable conditions for NSP and phytate degradation in the digestive tract affect the efficacy of the exogenous enzymes to degrade substrates in the digestive tract limit their efficacy and hinder a higher digestibility of P and N in pigs. These are the drawbacks that influence the digestion and absorption of P and N in pigs (Figure 4).

**Figure 4.** The limiting factors influence the digestion and absorption of P and N in pigs

NSP = non-starch polysaccharides

- The pigs do not secrete or secrete insufficient amount of enzymes to degrade NSP and phytate that encapsulate P, protein and amino acids in feedstuffs.
- The short retention time and the unfavourable conditions for NSP and phytate degradation in the digestive tract affect the efficacy of the exogenous enzymes in pigs.
2. Potential and limitations of cereals

2.1. Cereal composition

Cereals contain a high level of carbohydrates (65-75%) and a low level of protein (7-12%) and lipids (2-6%) (Haard, 1999; Wrigley, 2010). As an important energy source in pig diets, starch accounts for about 60% in barley, 64% in wheat and 73% in corn (Evers et al., 1999).

Based on the Osborne's classification, cereal protein can be divided into 4 groups: albumins (water soluble protein), globulins (saline soluble protein), prolams (alcohol soluble protein) and glutelins (insoluble protein) (Wrigley, 2010). Albumins and globulins are normally grouped together as the soluble proteins, while prolams and glutelins are considered to be the insoluble proteins. Soluble proteins account for about 20% of total protein in cereal grains and are located in the endosperm, the aleurone and the embryo tissues (Evers et al., 1999). Prolamins account for about 40% and 45% of total N in wheat and barley, respectively, and about 50% of total N in maize (Doll and Welch, 1984). In fact, the high proportion of prolams with low contents of the indispensable amino acids causes the low nutritional value of cereal proteins (Doll and Welch, 1984).

Cereals are rich sources of P (Cordain, 1999). Among cereals, barley has the highest total P content (4.2 g/kg DM) and rye the lowest (3.6 g/kg DM) (Steiner et al., 2007). Phosphorus is stored in 2 forms: (1) the organically bound P-salts of phytic acid (phytate P) and (2) non-phytate P (Waldroup, 1999). On average, 2/3 of the total P content in cereals is stored in the phytate form. The main storage site of P is in the aleurone cells, so the amount of total P bound to phytate in cereal by-products (85 – 90%) is higher than in the whole grains (Steiner et al., 2007).

The composition of cell wall in cereal grain are cellulose, hemicelluloses, pectins and lignin (Haard, 1999). The hemicelluloses or NSP, e.g. arabinoxylans and (1-3, 1-4)-β-glucans are the principal constituents of the cell walls of the endosperm and the aleurone cell. For example, the cell walls of barley endosperm are made up of 70-75% β-glucan and 20% arabinoxylan and protein (Edney et al., 2010; Fincher, 1975).

2.2. Nutritive value

Although normally considered to be the main energy sources, cereals also contribute about 40 to 50% of the dietary crude protein in growing-finishing pig because the cereal part often constitutes above 70% in the diet (Sauber and Owens, 2001). The AID of N of cereals can vary in a broad range: wheat (71-87%), maize (61-89%) and especially barley
(53-83%) (Table 1). The first and second limiting amino acids in most cereals are Lys and Thr, except maize which is limiting in both Lys and Trp. In general, the AID of amino acids vary according to the cereal type (wheat > corn > triticale > barley > oats > rye) (Sauer and Ozimek, 1986).

Among cereals, wheat, triticale and barley have a higher ATTD of P (> 30 %) in pigs (Kiarie and Nyachoti, 2010) due to their high endogenous phytase activity (Selle and Ravindran, 2008). The ATTD of P (50%) was highest for wheat (50%) and lowest for maize (18%) (Kiarie and Nyachoti, 2010). However, the ATTD of P of cereals varies over a wide range, e.g. maize (9-29%), sorghum (20-28%) and wheat bran (20-28%) (Kiarie and Nyachoti, 2010). This is due to the differences in the phytate P content and the endogenous phytase activity between cereal types and cultivars (Jongbloed and Kemme, 1990). Moreover, even the phytate content within the grains depends on the maturation stage (Jongbloed and Kemme, 1990). For example, cereals have the highest phytate P content at ripening time. However, the endogenous phytase in cereals is activated to degrade phytate during germination, so the digestibility of cereal P is increased during this period. Table 2 summarizes the ATTD of P in various cereal diets without and with microbial phytase supplementation in recent studies.
### Table 1. Crude protein content (g/kg DM) and digestibility of protein and amino acids (%) of various cereals in pigs

<table>
<thead>
<tr>
<th>Cereal</th>
<th>M</th>
<th>CP</th>
<th>AID N1</th>
<th>SID N4</th>
<th>Apparent ileal digestibility (AID) of Indispensable amino acids</th>
<th>Reference</th>
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<td></td>
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<td>81</td>
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<tr>
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<td></td>
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* M = Method used to measure digestibility: 1 = T-cannulated; 2 = re-entrant cannulation; 3 = ileo-rectal anastomosis; 4 = faecal collection, 5 = slaughter technique.
2 CP = Crude protein
3 AID N = apparent ileal digestibility of N.
4 SID N = standardized ileal digestibility of N.
5 Based on 7 studies from 1976-1983.
6 Based on 5 studies from 2007-2010.
7 Based on 6 studies from 1977-1981.
8 Based on 4 studies from 1977-1983.
9 Data were mean of results in 4 studies from 1980-1989.
10 Data were mean of results in 26 studies from 1977-2010.
*The value is SID of amino acids of wheat for pigs.
### Table 2. Apparent total tract digestibility (ATTD) of P (%) of various cereal diets without and with microbial phytase supplementation

<table>
<thead>
<tr>
<th>Diet¹</th>
<th>Total P (g/kg DM)</th>
<th>Phytate P (g/kg DM)</th>
<th>Endogenous phytase activity (FTU/kg DM)</th>
<th>Added microbial phytase activity (FTU/kg)</th>
<th>ATTD of P²</th>
<th>Reference</th>
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<td>3.5</td>
<td>2.4</td>
<td>153</td>
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<tr>
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<tr>
<td>Triticale-rye-SBM</td>
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<tr>
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</tr>
</tbody>
</table>

¹SBM = soybean meal
²-E/+E = without or with microbial enzyme supplementation
2.3. **Anti-nutritional factors**

Anti-nutritional factors are substances that interfere and depress digestion, absorption and utilisation of nutrients in animals (Huisman and Tolman, 1992). NSP and phytate are 2 of the anti-nutritional factors in cereals that are discussed in detail in this section.

**Non-starch polysaccharides (NSP)**

NSP belong to the polysaccharide group due to their high molecular size (degree of polymerization, ≥10) (Englyst et al., 2007). NSP are also considered to be the non-digestible carbohydrates which “cannot be degraded by host endogenous enzymes, but potentially can be degraded by microbial fermentation” (Bach Knudsen et al., 2012).

![Figure 5. Heterogeneity in structures of wall polysaccharides in plants. The backbone structures of these polysaccharides are based on (1,4)-β-linked monosaccharides in the case of cellulose, xyloglucan, (1,3;1,4)-β-glucan, heteroxylan (also arabinoxylan) and heteromannan. Adapted from Burton et al. (2010) ](image)

NSP mainly consist of cellulose, pentosans (arabinoxylans, xylans), β-glucans and pectins. In wheat and triticale, the cell walls in the starchy endosperm and the aleurone cells are mainly made of arabinoxylans (60-70%), whereas in barley, the cell walls consist predominantly of β-glucans and only 20% arabinoxylans (Fincher and Stone, 1986) (Figure 5).

The molecular structure of arabinoxylans comprises a linear (1-4)-β-xylan backbone with 2 pentoses: arabinose and xylose (Perlin, 1951) (Figure 5). Most of the arabinoxylans in cereals are insoluble, because they bind to the cell wall by the alkali-labile ester-like cross links (Mares and Stone, 1973). When the arabinoxylans are not trapped inside the cell wall
matrix, they become highly viscous and absorb water tenfold their weight (Mares and Stone, 1973).

The molecular structure of β-glucans is less complex compared with arabinoxylans (Figure 5). The β-glucans are made of glucose units that are linked together by β-(1-3) and β-(1-4) linkages into a linear chain (Bengtsson et al., 1990). Because of their high solubility, the β-glucans are degraded easily by the microorganisms colonising the distal segment of the small intestine (Bach Knudsen and Hansen, 1991). Therefore, the NSP in oat and barley (high β-glucan) are degraded to a greater extent compared with the NSP in wheat (high arabinoxylan). In fact, the fermentation of NSP in the large intestine can contribute from 10 to 24% of the energy for maintenance (Bach Knudsen and Hansen, 1991).

However, the degradation of the complex structure of NSP requires a combination of various enzymes that are not secreted by the pigs (Bedford, 1995). Moreover, the large polymers in NSP can entangle in large networks or mesh-like structures and create a high viscous solution (Bedford, 1995). Consequently, the high viscosity of NSP reduces the digestive capacity, the digestion rate, and the feed passage rate and thereby affect the nutrient digestion and absorption in the digestive tract of pigs (Bedford, 1995). Moreover, NSP encapsulate the essential nutrients like protein and minerals in their rigid matrix. NSP, therefore, become the physical barriers that prevent the digestion and absorption of these essential nutrients (Nortey et al., 2007a). Increasing the dietary NSP content also reduced the AID of indispensable amino acids (Lys, Met and Thr) (Nortey et al., 2007a) and the energy digestibility (Nortey et al., 2007a; Wenk, 2001).

In general, NSP encapsulate P and protein in the grain matrix. The degradation of the complex structure of NSP to release P, protein and amino acids for absorption is one of the challenges to enhance the digestibility of P and N of cereals in pigs.

Phytate

Phytic acid is the common name of myo-inositol 1, 2, 3, 4, 5, 6 hexakis di-hydrogen phosphate, whereas phytate is the term for the salt of phytic acid (Pallauf and Rimbach, 1997). The myo-inositol ring and the 6 symmetrical phosphate groups results in the chemical stability of phytate. Moreover, 6 phosphate groups of phytic acid carry 12 negatively charged ions that attract the positively charged molecules such as proteins and mineral cations (Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}) to form the insoluble complexes (Thompson Jr, 1978) (Figure 6).
Up to 75% of the total P content of grains is stored in the form of phytate (Ravindran 1996). The location and composition of phytate differ between cereals and oil seeds. Phytate is found in the bran (aleurone layer, testa and pericarp) of wheat, barley and triticale, but in the germ of maize, in the cotyledon of legumes and in the endosperm of linseeds (Pallauf and Rimbach, 1997). On average, the phytate P content (as percentage of total P) is different between various cereals: maize (75%) > triticale (72%) > wheat (70%) > barley (63%) > rye (60%) > oats (59%) (Weremko et al., 1997).

**Figure 6.** Phytate forms various complexes with protein and minerals. Adapted from Ruckebusch et al. (2013)

Phytate forms complexes with minerals at neutral pH of the small intestine and thereby reduces the availability of minerals for absorption (Woyengo and Nyachoti, 2013) (Figure 6). Phytate also increases the endogenous loss of minerals by increasing the secretion of minerals in the small intestine or by reducing the absorption of minerals from the small intestine (Woyengo and Nyachoti, 2013). As discussed in section 1.2, pigs do not secrete sufficient amounts of phytase to degrade phytate, so the high levels of phytate in the pig diet lead to a low digestibility of minerals like P and Ca (Selle and Ravindran, 2008). Consequently, the low digestibility of P results in the excessive P excretion in faeces.
and influences both animal productivity and environmental pollution. Selle et al. (2012) state that phytate binds protein and amino acids in different insoluble complexes (Figure 6) and thereby affect their digestion and absorption in the digestive tract negatively:

1. At pH below the protein isoelectric points, phytate forms the binary protein-phytate complexes by the electrostatic attraction between the polyanionic phytate molecules and the positively charged proteins. Consequently, the binary protein-phytate complexes resist pepsin digestion.

2. At pH above the protein isoelectric points, phytate forms the ternary protein-phytate complexes by linking negatively charged proteins with phytate via a cationic bridge (mostly Ca$^{2+}$). This reduces the absorption of not only protein and amino acids but also minerals in the small intestine.

3. According to the Hofmeister series, phosphates (HPO$_4^{2-}$) is a kosmotrope that can stabilise protein. Therefore, phytate, with 6 phosphates in the structure, also has strong kosmotrope properties that indirectly reduce the protein solubility and thereby limits the absorption of protein in the small intestines.

The isoelectric point of cereal protein (5.90 to 6.45) is higher than that of soybean meal (4.1) and other oilseed meal (4.70-5.50) (Selle et al., 2012). This implies that phytate may bind with cereal proteins as well as oilseed proteins to form the binary protein-phytate complexes in the stomach (pH 3-5). However, because of a higher isoelectric point of cereal proteins, the binary complex of phytate with cereal protein will be more resistant in the upper part of the small intestine (pH 5-6) compared with oilseed meal.

Woyengo and Nyachoti (2013) proposed the mechanism behind the negative effect of phytate on the ileal nutrient digestibility and the endogenous nutrient losses as presented in Figure 7. In summary, phytate can form the insoluble complexes with nutrients (protein, amino acids and minerals), digestive enzymes and endogenous nutrients in the stomach and the small intestine (Woyengo and Nyachoti, 2013). This reduces the digestive enzyme activities and the re-absorption of endogenous nutrients in the digestive tracts and consequently increases the secretion of pepsin and HCl in the stomach and of digestive enzymes in the small intestine (Woyengo and Nyachoti, 2013). Phytate therefore leads to an increase in the ileal endogenous flow of nutrients and a decrease in the true ileal nutrient digestibility (Woyengo and Nyachoti, 2013).
Overall, the anti-nutritional effect of phytate on nutrient digestion and absorption, especially P and N emphasizes the importance of phytate degradation to enhance the nutritive value of cereals.

Figure 7. Proposed mechanism of action of dietary phytic acid on ileal digestibility and endogenous losses of nutrients (Woyengo and Nyachoti, 2013)

2.4. **Endogenous enzymes**

The activity of endogenous enzymes varies in cereal grains and is affected by many factors such as cereal cultivar, environmental conditions during cultivation, pre-harvest sprouting, storage conditions and processing conditions (Poutanen, 1997).

The distribution of enzymes is not uniform within the cereal grain, as most of the enzymes are stored in the outer layers of grains (the aleurone and bran layers) and in the germ (Poutanen, 1997). The aleurone cells are responsible for the synthesis and release of different enzymes to break down the complex cell wall in order to gain access to the stored nutrients in the endosperm (Edney et al., 2010). The endogenous enzyme activities are much lower in the endosperm compared with the bran and the high fibre parts of cereal grains (Poutanen, 1997). When the grains are dry stored, the endogenous enzyme
activities in the mature grains are low (Evers et al., 1999). Most of the endogenous enzymes are released or synthesised during germination of cereal grains. This section focuses on the 3 main groups of enzymes in cereals: protein degrading enzymes, NSP degrading enzymes and phytate degrading enzymes.

**Protein degrading enzymes**

The cereal proteases can be divided into the 4 main groups: serine proteases, aspartic proteases, cysteine proteases and metalloproteases.

Serine proteases (EC 3.4.21) such as trypsin and chymotrypsin have their optimum pH between 7.5 to 10.5 (Delcour and Hoseney, 2010). Two serine proteases found in barley grain are serine endoprotease-1 (SEP-1) and Hordolisin (Jones, 2005). Serine proteases are located in the germinating embryos, so they are mostly responsible for protein metabolism (processing or turnover) during the early stages of grain development (Delcour and Hoseney, 2010).

Wheat and barley grains also have aspartic proteases (EC 3.4.23) which have 2 aspartic acid residues in their active site and the acidic pH optimum (Delcour and Hoseney, 2010). The enzymes belonging to this group are pepsin, chymosin, rennin and cathepsin D, which target the peptide bonds between amino acid residues with large hydrophobic side chains (Delcour and Hoseney, 2010). Aspartic proteases have been found in both the aleurone layer and the starchy endosperm cells (Zhang and Jones, 1995).

Cysteine proteases (EC 3.4.22), also known as “cysteine-type” peptidases, play an important role in degrading storage protein of both wheat and barley during germination (Jones, 2005). To function properly, cysteine proteases require a cysteine residue present in their active site (Jones, 2005), and they can function in a broad pH range with the optimal pH below 7.0 (Delcour and Hoseney, 2010). For example, barley cysteine proteases have an optimum pH at 4.8 (Zhang and Jones, 1995). Moreover, cathepsin-like cysteine proteases located in the endosperm and in the aleurone cells of barley support the protein deposition and subsequently the mobilisation of protein during germination (Martinez et al., 2003).

Metalloproteases require the presence of a metal ion such as zinc at their active site to function properly (Jones, 2005). The metalloproteases in barley have their optimal pH at 7 to 8 (Jones, 2005), and the activities of metalloproteases have been detected in the aleurone, embryo and starchy endosperm tissues (Zhang and Jones, 1995). Thus, the
enzymes play an important role in mobilizing the stored protein during germination and malting process (Jones, 2005).

It is important to emphasize that endogenous protease inhibitors also exist in the developing and mature grains as well as the germinating grains. The endogenous protease inhibitors can hinder or even switch off the activity of proteases and thereby inhibit protein degradation (Christensen, 2013b; Davy et al., 1999; Jones, 2005). Additionally, cereal grains also use the endogenous inhibitors as a defence mechanism to target specifically the microbial proteases and the proteolytic enzymes secreted in the digestive tract of pigs (Mikola and Enari, 1970).

*Non-starch polysaccharide degrading enzymes*

Endo-1,4-β-xylanase (EC 3.2.1.8), also termed endoxylanase or xylanase, hydrolyses the internal β-(1, 4)-linkages in the backbone of arabinoxylans into oligosaccharides, xylobiose and xylose (Delcour and Hoseney, 2010; Dornez et al., 2009). Xylanases are distributed unevenly in the cereal grain, and the xylanase activity seems to increase from the starchy endosperm to the pericarp (Dornez et al., 2009). The xylanase activity also varies between different cereals (oat > maize > barley > rye > wheat) (Preece and MacDougall, 1958). The xylanase activity of various grains is presented in Table 3.

**Table 3.** Xylanase activity (EU/g) in bran and flour derived from different cereals (modified from Dornez et al. (2009))

<table>
<thead>
<tr>
<th>Cereal species</th>
<th>Flour fraction</th>
<th>Bran fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common wheat</td>
<td>0.06</td>
<td>0.54</td>
</tr>
<tr>
<td>Oats</td>
<td>0.49</td>
<td>0.89</td>
</tr>
<tr>
<td>Barley</td>
<td>0.33</td>
<td>1.22</td>
</tr>
<tr>
<td>Rye</td>
<td>0.07</td>
<td>0.29</td>
</tr>
</tbody>
</table>

1 EU = enzyme unit. One EU is the amount of enzyme needed to increase the extinction at 590 nm by 1.0 per hour of incubation under the conditions of Xylozyme-AX assay.

Interestingly, recent studies revealed that cereals also contain microbial xylanases produced by the microorganisms on the surface of grain kernels (Dornez et al., 2009; Dornez et al., 2006). Over 90% of the wheat xylanase activity may be contributed by microbial xylanases (Dornez et al., 2006).

Another important NSP-degrading enzyme in cereal grain is β-glucanase. Barley has t3 endo-β-glucanases (Ballance et al., 1976; Hrmova et al., 1999):

1. **Endo-β-1, 4-glucanases** or cellulases (EC 3.2.1.4) hydrolyse the β-1,4-linkages.
2. **Endo-β-1,3-glucanases** cleave the β-1,3-linkages.
(3) (1,3; 1,4)-β-glucan endohydrolases (EC 3.2.1.73) degrade both β-1,3- and β-1,4- linkages.

These enzymes are present in various locations of the grain and are associated with different stages of grain development and germination. Endo-β-1,4-glucanases are mainly present in the hull and endosperm (Ballance et al., 1976). Besides β-glucan, cellulose is the main substrate for endo-β-1,4-glucanases which explains their other name - cellulases (Ballance et al., 1976; Høj and Fincher, 1995). Endo-β-1,3-glucanases are mainly located in the embryo and the scutellum and to a limited extent in the hull of mature grains (Ballance et al., 1976). The aleurone cells synthesise endo-β-1,3-glucanases de novo, so their activity increases rapidly during germination (Bennett and Chrispeels, 1972). Moreover, endo-β-1,3-glucanases can disrupt the fungal cell walls, so they are associated with the protective strategy of the grains against the external pathogens during germination (Høj and Fincher, 1995). The mature barley grains, however, have no (1,3; 1,4)-β-glucan endohydrolase. The (1,3; 1,4)-β-glucan endohydrolase is synthesised during germination and mainly stored in the endosperm. Thus, (1,3; 1,4)-β-glucan endohydrolase hydrolyses the linkaged β-glucan of the cell walls and opens up the stored nutrients for other enzymes (Høj and Fincher, 1995). A recent study of Ribeiro et al. (2011) revealed a broad range of cellulase and (1,3; 1,4)-β-glucan endohydrolase from less than 60 to more than 1300 U/kg in various barley varieties. The same study observed that the endogenous β-glucanase of barley decreased the digesta viscosity of chicken to the same extent as the added exogenous β-glucanase (Ribeiro et al., 2011). Thus, solely endogenous β-glucanase in barley is capable of degrading the NSP and improving the nutritive value of cereal grains.

Phytate degrading enzymes

Cereals and cereal by-product have high endogenous phytase activities compared with legume seeds (Eeckhout and Depaepe, 1994). Except for maize (0-46 U/kg DM), oats (0-108 U/kg DM) and sorghum (0-76 U/kg DM), the endogenous phytase activities of various cereals are in the range of 408-6127 U/kg DM (barley < wheat < triticale < rye), and the higher value is detected in cereal by-products with 1180-5345 U/kg DM (Eeckhout and Depaepe, 1994). This is because endogenous phytase is predominantly located in the aleurone layer and scutellum of cereal grains, so cereal by-products like wheat and rye bran normally have high phytase activities (Steiner et al., 2007). According to Peers (1953), 40% of the total endogenous phytase in wheat is located in the aleurone layer, 34% in the
The pigs do not secrete or secrete insufficient amount of enzymes to degrade NSP and phytate.

The short retention time and the unfavourable conditions for NSP and phytate degradation in the digestive tract affect the efficacy of the exogenous enzymes in pigs.

NSP and phytate encapsulates P, protein and amino acids in the grain matrix.

High amount of dispensable amino acids and limited amount of some indispensable amino acids.

Endogenous enzymes in cereals are capable of degrading NSP and phytate.

Overall, cereals constitute above 70% of the pig diets and thereby contribute about 40-60% of P, protein and amino acids to meet the pigs’ requirement. However, NSP and phytate embed P and protein tightly and thereby hinder an increased digestibility of cereal nutrients. Moreover, the high amount of dispensable amino acids and the limited amount of some indispensable amino acids lead to a high content of amino acids that are deaminated and excreted as urea in urine. Therefore, the cereal part seems to be one of the drawbacks for further enhancement in the P and N digestibility and utilization in pig diets (Figure 8). However, the endogenous enzymes in cereals are capable of degrading NSP and phytate (Figure 8). Hence, taking advantages of this enzyme source may be effective in enhancing the digestibility of P and N of cereals.

**Figure 8.** The potential and drawback of cereals regarding P and N utilisation in pigs

NSP = non-starch polysaccharides.
3. Effect of various feed processing on enhancing digestibility of P and N in pigs

3.1. Grinding and rolling

Grinding reduces the grain particle size, and thereby increases the surface area and the substrate accessibility for the microbial and digestive enzymes (Lahaye et al., 2008; Mansfield et al., 1999; Rowe et al., 1999). Decreasing the average particle size of ground wheat from 1432 µm to 460 µm increased the particles’ surface area 53% (Mavromichalis et al., 2000). However, it is important to keep in mind that even small particles can contain individual starch granules (or P and protein) which are bound tightly within the endosperm matrix and are inaccessible for the digestive enzyme (Rowe et al., 1999).

When decreasing the particle size of diets from 700 to 400 µm, the ATTD of P was increased by 15%, and the faecal P excretion was reduced by 12% (Oryschak et al., 2002). In fact, grinding per se cannot reduce the phytate content in cereals, but the ATTD of P was increased in pigs because grinding disrupted the grain matrix and provided the substrate access for the phytate-degrading enzymes.

Grinding enhanced the ATTD of N and reduced the faecal N excretion in pigs fed wheat (Laurinen et al., 2000; Mavromichalis et al., 2000), barley (Laurinen et al., 2000; Nasi, 1992; Oryschak et al., 2002), maize (Healy et al., 1994; Wondra et al., 1995) and sorghum (Healy et al., 1994; Owsley et al., 1981). The reduction in feed particle size improves the AID of N to a greater extent than the ATTD of N in pigs fed barley (Oryschak et al., 2002), wheat (Sauer et al., 1977) and sorghum (Owsley et al., 1981). It seems that grinding enhanced the N digestion and absorption in the small intestine (Oryschak and Zijlstra, 2002). However, the reduction in feed particle size increased the urinary N excretion in pigs, even though the total N excretion was decreased by 4% (Oryschak et al., 2002; Owsley et al., 1981). This implies that a reduction in particle size shifted the N excretion from faeces to urine which consequently may increase the ammonia emission.

3.2. Liquid feeding

In recent years, liquid feeding has been widely applied for at least 30% of pigs in Europe (Missotten et al., 2010) and for 40% of the grower-finishers pigs in Denmark (Jakobsen, 2015). Liquid feeding includes 2 types: (1) non-fermented liquid feed where feed is mixed with water or other fluids before feeding and (2) fermented liquid feed where feed and water (fluids) are mixed and stored for a certain time period before feeding (Canibe and Jensen, 2003). Various reviews (Canibe and Jensen, 2012; Missotten
Missotten et al., 2010; Plumed-Ferrer and Von Wright, 2009) discussed several advantages of fermented liquid feeding. Two of these advantages are:

1. Increased nutrient digestibility, e.g. the digestibility of P and N
2. Reduced contents of anti-nutritional factors, e.g. NSP, phytate, trypsin inhibitors, tannins and saponin, α-galactosides.

The mechanisms behind the effect of liquid feeding on enhancing nutrient digestibility may be due to the formation of organic acids, the activation of endogenous enzymes in cereals and the release of enzymes from microorganism during fermentation (Canibe and Jensen, 2012; Missotten et al., 2015). Thus, cereals may go through a “pre-digestion” process before feeding which release P and protein encapsulated in the grain matrix and makes these nutrients more available for pigs. This effect of liquid feeding may compensate for the short retention time for NSP and phytate degradation along the digestive tract as described in section 1.2. Fermented liquid feeding also increases the digestive capacity of pigs as shown in a greater villus length and a greater villus/crypt ratio (Scholten et al., 2002). Various studies demonstrate that liquid feeding hydrolyses phytate (Blaabjerg et al., 2010b; Blaabjerg et al., 2015; Liu et al., 1997; Lyberg et al., 2006) and NSP (Jorgensen et al., 2010; l’Anson et al., 2013; Lyberg et al., 2006) in cereals. However, the reported effects of liquid feeding on the P and N digestibility is inconsistent, as liquid feeding enhanced the ATTD of P of wheat-barley (Blaabjerg et al., 2010b; Lyberg et al., 2006), wheat-barley-maize (Blaabjerg et al., 2015), but not of maize and soybean meal (Nitrayová et al., 2009; Pedersen and Stein, 2010). This may be due to the negligible endogenous phytase activity in maize and soybean meal compared with wheat and barley. The AID of N was also increased in fermented wheat and barley (Lyberg et al., 2006; Sholly et al., 2011), but not in maize and soybean meal (Hong et al., 2009).

The extent of the improvement in the digestibility of P and N caused by liquid feeding varies between the different growth stages of pigs (weaner pigs > growing-finishing pigs) and depends on several factors (Canibe and Jensen, 2012):

1. Diet composition, e.g. the activities of endogenous enzymes and the content of phytate and NSP in cereals
2. Fermentation conditions, e.g. pH, temperature, water ratio and microorganism
3. Feed additive supplementation, e.g. microbial enzymes and organic acid

Moreover, there may be several disadvantages associated with liquid feeding. One of the main problems that occur during the fermentation process is the microbial
decarboxylation of the free amino acids in feedstuffs and the free synthetic amino acids added to the feed, especially Lys (Canibe and Jensen, 2010; Niven et al., 2006). Consequently, the decarboxylation process transforms the free amino acids to biogenic amines such as cadaverine from L-Lys, histamine from His, putrescine from Met, tyramine from Tyr and tryptamine from Trp (Spano et al., 2010). Biogenic amines have many critical functions, e.g. as precursors of the synthesis of hormone, alkaloids, nucleic acids and proteins (Premont et al., 2001), but their formation also causes the irreversible loss of amino acids in pigs (Canibe and Jensen, 2010).

3.3. **Exogenous enzymes addition**

Exogenous enzymes have been added to animal feeds since the 1950s. They play a major role in enhancing the nutrient digestibility and utilisation and in reducing the nutrient excretion (Adeola and Cowieson, 2011). Phytase constitutes 60% of the global feed enzyme market, while the NSP-degrading enzymes or carbohydrates – comprising 2 dominant enzymes: xylanase and glucanase – hold 30% of the market (Adeola and Cowieson, 2011). Other enzymes such as proteases, lipases and amylases have a small share of the feed enzyme market, but their effects and potentials are expected to attract more attention in the near future. Recent reviews have already provided a detailed knowledge on the exogenous enzymes and their effects on the enhancement in the nutrient digestibility in pigs, e.g. Bedford (2000), Selle and Ravindran (2008) and Adeola and Cowieson (2011).

Many studies have demonstrated the effect of microbial phytase on enhancing the P digestibility in pigs (Selle and Ravindran, 2008). Diet composition and processing clearly affect to which extent the P digestibility is enhanced by the addition of microbial phytase. Microbial phytase can increase the ATTD of P up to 60-74% in cereal diets without the inorganic phosphate supplementation (Almeida and Stein, 2012; Atakora et al., 2011; Kim et al., 2008; Poulsen et al., 2007). The maximum increase in the ATTD of P could be up to 39% points in maize diets (Almeida and Stein, 2012; Emiola et al., 2009; Jolliff and Mahan, 2012; Kerr et al., 2010) but only 23% points in barley-wheat diets (Grela et al., 2011; Htoo et al., 2007; O’Doherty et al., 2010; Poulsen et al., 2007). This difference is mainly due to the negligible endogenous phytase activity of maize compared with the much higher phytase activity in barley and wheat. Microbial phytase increases the P digestibility to a greater extent in the low endogenous-phytase diets compared with the high endogenous-phytase diets. However, even with microbial phytase, 36-40% of the P intake is still undigested and
excreted to faeces (Almeida and Stein, 2012; Atakora et al., 2011; Kim et al., 2008; Poulsen et al., 2007) which implies the possibility of further improvement.

The reported effects of carbohydrases, especially xylanase and glucanase, on enhancing the nutrient digestibility and utilisation are inconsistent in pigs (Adeola and Cowieson, 2011). The main role of carbohydrases is to break the complex structure of arabinoxylans and β-glucans in the grain matrix to increase the accessibility of the other enzymes to the stored nutrients. These processes mimic the germination process inside the grains where carbohydrases hydrolyse the cell wall of the aleurone and endosperm cells. These processes require not only 1 but a combination of various carbohydrases. The effects of carbohydrase supplementation on the enhancements in the N and amino acids are associated with: (1) the reduction in the NSP content that leads to a decrease in endogenous protein and amino acid loss and (2) the increase in the accessibility of the proteolytic enzymes to protein and amino acids (Adeola and Cowieson, 2011). The effects of carbohydrase addition on enhancing the amino acid digestibility are limited and inconsistent in pigs (Kiarie et al., 2012; Li et al., 1996; Nortey et al., 2008; Willamil et al., 2012). Overall, the addition of carbohydrases only influences the digestibility of nutrients indirectly, because they provide access for other enzymes (phytase, protease and amylase).

Proteases have been added to poultry diets as a single enzyme but to pig diets in a combination with other enzymes. Limited number of studies have investigated the effect of proteases, but the reported effects on the N and amino acid digestibility in pigs seem to be inconsistent (Adeola and Cowieson, 2011). Thus, more research is needed regarding the potential effect of proteases and the interaction with other enzymes on enhancing the digestibility of N and amino acids of different protein sources in pig.

In theory, the combination of various enzymes used to target the different substrates may maximise the effects of the individual enzymes on the digestibility of P and N. However, less information is available on the effect of enzyme combination on nutrient digestibility in pigs compared with poultry. Nortey et al. (2007b) observed a synergistic effect of using carbohydrases in combination with phytase on the enhancement in ATTD of P. Nevertheless, several studies observed no additive increase when using an enzyme combination compared with the addition of the individual enzyme (Atakora et al., 2011; Mc Alpine et al., 2012; Olukosi et al., 2007).
3.4. High moisture storage

After harvesting, the storage of cereals is the first process that may influence the quality of cereals. At least 2 “living entities” exist in the stored grains: 1) the grain themselves, and 2) the microorganism colonizing them (Choct and Hughes, 1997). These 2 entities cause various degrees of physical, chemical and biological changes during storage (Choct and Hughes, 1997). The moisture content together with the temperature and the oxygen concentration influence the activation of endogenous enzymes in cereals and the microbial growth (Choct and Hughes, 1997). Thus, the moisture content is normally restricted to 13% to guarantee a safe storage of cereals like barley, wheat and maize (Laca et al., 2006). In temperate climates, cereals are harvested at a high moisture content to protect the grains from wet weather conditions that result in the mould infection of the grain in the field (Druvefors and Schnurer, 2005; Karunakaran et al., 2001). Thus, it requires high energy expenditure for drying to achieve the 13% moisture level in harvested cereals (Passoth et al., 2009).

Several alternative storage methods may be used to replace dry storage such as high moisture storage, ensiling, airtight storage and high moisture airtight storage. The terms used for these storage methods are inconsistent in the literature and therefore difficult to differentiate. This section defines and unites the terms used for each method.

Ensiling is the process of storing high moisture forages or other materials (entire crop or only part of a crop) anaerobically to prevent the decomposition by the aerobic microorganisms and the endogenous enzymes (Woolford and Pahlow, 1997). Ensiling involves the acidification of the crops by organic acids produced by the microbial fermentation, principally lactic acids (Wilkinson et al., 2003). Thus, silage is “an acidic, fermented, stored product from an agricultural crop” (Wilkinson et al., 2003).

High moisture storage has a similar storing mechanism like ensiling. However, it is limited to the preservation of high moisture grains and grain by-products in the anaerobic condition. The common cereals that are stored in high moisture conditions are barley in Europe and Western Canada and maize in North America (Buchanan-Smith et al., 2003). Cereal grains are normally stored in the upright airtight oxygen-limiting sealed structures or in the unsealed upright/horizontal structure (Buchanan-Smith et al., 2003). The fresh grains or the dried grains reconstituted with water are stored at a moisture content of 20-35% and for a period of no more than the time interval between harvests (up to 12 months).
High moisture grain storage has several advantages and disadvantages as presented in Table 4 (Buchanan-Smith et al., 2003).

**Table 4. Advantages and disadvantages of high moisture grain storage compared with dry storage (modified from Buchanan-Smith et al. (2003))**

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher yields due to earlier harvesting of grains</td>
<td>Reduce the time interval available for harvesting grains</td>
</tr>
<tr>
<td>Save drying cost and reduce fuel consumption</td>
<td>Greater power required to process after storage</td>
</tr>
<tr>
<td>Increase flexibility to harvest grains</td>
<td>Loss of flexibility to sell the grains on the cash market</td>
</tr>
<tr>
<td>Allow rapid handling of large volumes of grains at harvest</td>
<td>Loss of fluidity to move the grains</td>
</tr>
<tr>
<td>Reduce investment in processing equipment</td>
<td>Require considerable capital for storage facilities</td>
</tr>
<tr>
<td>High feeding value and even higher than dry grains</td>
<td>Improper storage could result in complete loss of feed</td>
</tr>
<tr>
<td>Reduce dustiness in feed processing and in livestock barns</td>
<td>Grain is liable to freeze in winter and attract flies in summer</td>
</tr>
</tbody>
</table>

Airtight storage is also termed “hermetic storage”, “sealed storage”, “sacrificial sealed storage” or “hermetic silo storage” (Villers et al., 2010). Airtight storage preserved the grains at intermediate moisture contents in the sufficient sealed structures with the modified atmosphere (by eliminating O₂ and increasing CO₂ concentration). The modified atmosphere can be achieved in several ways: (1) tight sealing or underground sealing and natural respiratory metabolism of the aerobic microorganisms in the grain mass that consume O₂ and produce CO₂ (similar to ensiling); (2) fumigating CO₂; (3) applying a significantly high vacuum (Villers et al., 2010).

High moisture airtight (HMA) storage is a combination of high moisture storage and airtight storage. HMA storage preserves the grains at a high moisture content in an airtight condition. Thus, HMA storage has similar advantages as mentioned in high moisture storage. From this point forward, the term HMA storage is used in this thesis to refer to all types of storage with similar conditions (high grain moisture content and airtight condition) but may be called by different names in previous studies.

At harvest, the moisture content in grains can be as high as 46% in barley, 25% in oats and 31% in wheat (Svihus et al., 1997). This condition is ideal to activate the endogenous enzymes and promote the microbial fermentation to degrade NSP and phytate in the grain matrix (Choct and Hughes, 1997). HMA storage was found to decrease the content of soluble dietary fibre and soluble β-glucan in barley and oat (Svihus et al., 1997). Kim et al. (2003) also found a decrease in soluble NSP, ADF and lignin and an increase in free sugars in wheat after HMA storage for 6 months. It is most likely that the various endogenous
glycanases in cereals degrade the complex polysaccharides into smaller sugars during HMA storage (Kim et al., 2003). The decrease in soluble NSP also changes the ratio of insoluble NSP and soluble NSP and thereby enhances the amount of digestible energy (DE) in wheat (Kim et al., 2003). This improvement in the DE content is more pronounced in wheat with high NDF and xylose contents (Kim et al., 2005). HMA storage also improved the amount of soluble protein in barley (Åman et al., 1990a) and maize (Baron et al., 1986). HMA stored grains enhanced the N digestibility in pigs fed wheat by 2% (Pieper et al., 2011) and 10% (Myer et al., 1986) and barley by 4% (Weltzien and Aherne, 1987). The content of phytate P in HMA stored maize (46% of total P) was lower compared with dry maize (64% of total P) (Abrams et al., 1976). Consequently, a higher average daily gain, serum P and bone ash percentage was observed in pigs fed the HMA stored maize (Abrams et al., 1976). Niven et al. (2007) found a higher soluble P content in HMA stored maize due to the increased release of P from phytate. HMA storage also increased the P digestibility in triticale from 23 to 55% (Pieper et al., 2011), in wheat from 26 to 48% (Pieper et al., 2011) and in barley from 23 to 38% (Weltzien and Aherne, 1987). In general, HMA storage seems not only to preserve the grains but also to improve the nutritive value of cereals by taking advantage of the endogenous enzymes in cereals and the microbial fermentation. However, there is a limited number of published studies investigate the effect of HMA storage in pigs. Moreover, many factors need to be studied to maximise the enhancement in the digestibility of N, amino acid and P by HMA storage, e.g. cereal type, moisture level, pre-processing before storage and storage time.

Overall, there are some limiting factors that hinder the improvement of P and N digestibility and utilisation in pigs (Figure 9). Firstly, NSP and phytate embed protein and bind P in the grain matrix which may hinder an increase in the digestibility of cereal P and N. Moreover, the high amount of dispensable amino acids and the limited amounts of some dispensable amino acids in cereals also led to a high content of amino acids that are deaminated and excreted as urea in urine. Secondly, the pigs do not secrete or secrete insufficient amounts of digestive enzymes to degrade the complex structure of NSP and phytate. Thirdly, the endogenous enzymes in cereals and the added microbial enzymes are capable of degrading NSP and phytate to release P and N for absorption. However, the short retention time and the unfavourable conditions for NSP and phytate degradation in the digestive tract affects the efficacy of endogenous enzymes in cereals and the added microbial enzymes on improving the P and N digestibility in pigs. Thereby, HMA storage of
cereals with exogenous enzymes may be a potential approach that activates the endogenous enzymes in cereals and the added microbial enzymes during storage and compensates for the short retention time in the digestive tract by “pre-digesting” NSP and phytate before feeding.
PhD thesis - Background

Figure 9. Three facets of phosphorus (P) and nitrogen (N) problem: The pig – Cereals – Feed processing

- NSP and phytate encapsulate P, protein, and amino acids in the grain matrix.
- High amounts of dispensable amino acids and limited amounts of some indispensable amino acids result in the high excretion of N in urine.
- Endogenous enzymes in cereals are capable of degrading NSP and phytate.

- The pigs do not secrete or secrete insufficient amounts of enzymes to degrade NSP and phytate.
- The short retention time and the unfavourable conditions for NSP and phytate degradation in the digestive tract affect the efficacy of the exogenous enzymes in pigs.

- HMA storage with exogenous enzymes activates the endogenous cereal enzymes and the added enzymes:
  - Compensate for the short retention time in the digestive tract
  - Degradate NSP and phytate before feeding
  - Enhance nutritive value of cereals in pigs

NSP = non-starch polysaccharides; HMA storage = high moisture airtight storage
Chapter III. **Hypothesis & Aims**

1. **Hypothesis**

   (1) High moisture airtight (HMA) storage of barley and triticale activates endogenous enzymes prior to feeding resulting in an enhanced phytate degradation and solubility of P, N and protein compared with dry storage.

   (2) Grain processing, grain moisture, storage time and cereal type affect the magnitude of the effect of HMA storage on increasing phytate degradation and P, N and protein solubility.

   (3) The addition of a combination of phytase, xylanase, β-glucanase and protease to barley and triticale during HMA storage will even increase the phytate degradation and the solubility of P, N and protein to a greater extent compared with no enzyme addition.

   (4) The solubility of P is the indicator to predict the digestibility of P of cereals in pigs.

   (5) The solubility of N and protein is the indicator to predict the digestibility of N and amino acids of cereals in pigs.

   (6) HMA storage increases the ATTD of P, the ATTD and the AID of N, and the AID of amino acids of barley in pigs compared with dry storage.

   (7) A further increase in the digestibility of P, N and amino acids is achieved by HMA storage of barley together with the added enzyme combination promoting the degradation of grain matrix.

   (8) The activated endogenous enzymes in HMA stored barley and the added enzyme combination will increase the ATTD of P, the ATTD and AID of N and the AID of amino acids of SBM when fed together with HMA stored barley.

   (9) The enhancement in ATTD of P by HMA storage with enzyme combination will reduce the need for dietary inorganic P supplement and P excretion to the environment.

   (10) The increased ATTD and AID of N and AID of amino acids by HMA storage with enzyme combination will improve the quality of barley protein and thereby reduce protein feedstuff supplementation and N excretion to the environment.
2. Aims

The main aims of the PhD study:

(1) To evaluate the effect of grain processing, grain moisture, storage time and enzyme combination on the phytate degradation and the P, N and protein solubility of barley and triticale during HMA storage.

(2) To define the optimal conditions for maximising the phytate degradation and the P, N and protein solubility in HMA stored rolled barley as affected by endogenous enzyme present in the barley and the enzyme combination.

(3) To determine the digestibility and balance of P in pigs fed the HMA stored barley (without or with the enzyme combination) compared with the dry stored barley (without or with the enzyme combination).

(4) To determine the digestibility and balance of P in pigs fed compound diets based on barley stored dry or HMA (without or with the enzyme combination) together with SBM.

(5) To determine the digestibility and balance of N in pigs fed the HMA stored rolled barley (without or with the enzyme combination) compared with the dry stored barley (without or with the enzyme combination).

(6) To determine the digestibility and balance of N in pigs fed the compound diets based on barley stored dry or HMA (without or with the enzyme combination) together with SBM.

(7) To investigate the effect of storage (HMA stored vs. dry stored) of barley without or with the enzyme combination on AID of amino acids in pigs fed barley alone and without crystalline amino acids.

(8) To investigate the effect of storage (HMA stored vs. dry stored) of barley without or with the enzyme combination on AID of amino acids in pigs fed barley together with SBM but without crystalline amino acids.

(9) To evaluate the relationship between the P solubility and the P digestibility of barley.

(10) To evaluate the relationship between the N and protein solubility and the N and amino acid digestibility of barley.
Chapter IV. **Methodology**

1. **Materials**

Cereals used in the current study, barley (winter cultivar, *Zephyr*) and triticale were collected from Sejet Plant Breeding (Horsens, Denmark). Barley and triticale (13% and 10% moisture, respectively) were cleaned separately using a sample cleaner MLN (Wintersteiger AG, Ried/i., Austria) to remove dust and unwanted particles. Half of the barley and triticale remained as whole grain (whole), whereas the other half was coarsely ground (rolled) by a roller mill (Skiold crushers KB 200, SKIOLD A/S, Sæby, Denmark).

The enzyme combination used in the study included 4 microbial enzymes:

1. Phytase or myo-inositol-hexakisphosphate β-phosphohydrolase (EC 3.1.3.8) produced by *Aspergillus niger* (Natuphos 5000G with a minimum of 500 FTU/g, BASF SE, Ludwigshafen, Germany). The used dose: 1000 FTU/kg of feed.

2. Xylanase or endo-1, 4-β-xylanase (EC 3.2.1.8), dried fermentation product of *Trichoderma reesei* (Danisco Xylanase 8000 G with a minimum of 8000 U/g, Danisco Animal Nutrition, Marlborough, United Kingdom). The used dose: 4000 U/kg of feed.

3. β-glucanase or Endo-1, 3 (4)-β-glucanase produced by *Trichoderma reesei* (ECONASE Barley P700 with minimum 700,000 BU/g, AB Vista, Marlborough Wiltshire, United Kingdom). The used dose: 17500 BU/kg of feed.

4. Protease – a granulated serine protease produced by submerged fermentation of *Bacillus licheniformis* microorganism (EC 3.4.21) (RONOZYME ProAct CT with minimum 75, 000 PROT/g, DSM Nutritional Products Ltd, Basel, Switzerland). The used dose: 15000 PROT/kg of feed.
Table 5. The experimental factors and the measured parameters

<table>
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<th>Factors</th>
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<th>HMA storage</th>
<th>Measured parameters¹</th>
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¹The solubility of P, N and protein was measured in barley (before and after storage). ATTD = Apparent total tract digestibility, AID = Apparent ileal digestibility.
2. Experiments

The PhD study included *in vitro* and *in vivo* studies as presented in Table 5. The *in vitro* study (Experiments 1) investigated the effect of HMA storage and enzyme combination on phytate degradation and the solubility of P and N of barley and triticale. The study also screened several factors that influence HMA storage such as grain processing, grain moisture, storage time. Based on the results of Experiment 1, Experiment 2 was conducted to define the optimum moisture level to maximising the phytate degradation and the solubility of P, N and protein of HMA stored rolled barley. The solubility of P, N and protein was used as the indicator to predict the P, N and amino acid digestibility in response to experimental factors. Storage of barley and triticale in Experiments I and II was studied in a small scale.

The *in vivo* study (Experiment 3) evaluated the effect of storage (dry storage vs. HMA storage at 35% moisture) and enzyme combination (without vs. with) on the ATTD and balance of P and N and the AID of N and amino acids in pigs fed barley diets or barley-SBM diets. Storage of barley in Experiment 3 was conducted in a medium scale. Details of the experiments were described in the manuscripts.

2.1. Storage of cereals – small scale

Cereals were either stored in 10-l covered plastic buckets (dry storage) at ambient temperatures (average 20°C in Experiment 1 and average 9°C in Experiment 2) or in vacuum packed plastic bags providing airtight conditions for barley and triticale at various moisture levels at 15°C (HMA storage).

Before HMA storage, the dry matter (DM) content of cereals was determined. Based on the DM content, the required amounts of water were added to the cereals to obtain the planned moisture levels. The procedure for HMA storage was as follows: 400 g of cereals was weighed in a plastic bag. After mixing thoroughly with demineralised water, the bag was sealed by a vacuum packaging machine (Webomatic I 22-D, Bochum, Germany). The enzyme combination of phytase, xylanase, β-glucanase and protease was added to the enzyme supplemented cereals at the same time as water. Samples in triplicate were randomly placed in an incubator (New Brunswick Scientific Model G25 Controlled Environment Incubator Shaker, GST Technical Sales, Edmonton, Alberta, Canada). The temperature of the incubator was measured every day at different positions, and the temperature was in the range from 14 to 18°C. The positions of samples in the incubator were changed randomly every week. The HMA stored cereals were collected at different
time points, whereas dry stored samples were collected at day 0 and day 49. After collection, samples were immediately frozen.

2.2. **Storage of cereals – medium scale**

Barley (winter cultivar, *Zephyr*) was ground by a roller mill (Skiold crushers KB 200, SKIOLD A/S, Sæby, Denmark) and stored (400 kg/bag) either at 13% moisture in plastic bags at an ambient temperature (average 9°C) (dry storage) or at 35% moisture in airtight bags (HMA storage) for 49 days. Before storage, rolled barley was supplemented without or with an enzyme combination. The HMA stored barley was prepared as follows: adding water (supplemented with or without enzyme combination) to rolled barley (13% moisture) to adjust the moisture content to 35%, sealing the bag and mounting a valve to fill up CO₂ regularly to secure airtight conditions during storage. Before storage (day 0) and after 49 days of storage, samples in triplicate were collected and immediately frozen to stop further degradation.
Chapter V. **Results**

The results of the PhD study were divided into 3 manuscripts:

**Manuscript I**: High moisture airtight storage of barley and triticale: Effect of moisture level and grain processing on nitrogen and phosphorus solubility.

**Manuscript II**: 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.

**Manuscript III**: Effects of high moisture airtight storage of barley with exogenous enzymes on protein and amino acid digestibility of barley fed to pigs alone or in combination with soybean meal.
Manuscript I

High moisture airtight storage of barley and triticale: Effect of moisture level and grain processing on nitrogen and phosphorus solubility

M. A. Ton Nu, K. Blaabjerg, and H.D. Poulsen

Submitted to Animal Feed Science and Technology for publication.
High moisture airtight storage of barley and triticale: Effect of moisture level and grain processing on nitrogen and phosphorus solubility

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ABSTRACT

The aim of this study was to evaluate the effect of storage time, grain processing (whole vs. rolled) and the combination of phytase, xylanase, beta-glucanase and protease on nitrogen (N) and phosphorus (P) solubility during high moisture airtight (HMA) storage of barley and triticale at various moisture levels (20, 23, 26 and 29% moisture) and to compare HMA storage of cereals with dry storage for 49 days. Dry stored barley and triticale (10% and 13% moisture, respectively) were kept in 10 L plastic buckets for 0 and 49 days. HMA stored cereals were kept in airtight bags (400 g per bag) at 15°C for 0, 14, 29 and 49 days. The cereals were dry stored or HMA stored in rolled or whole form without or with an enzyme combination. Samples in triplicate were measured for dry matter (DM), pH, N and P solubility, phytate P and total P. HMA storage of rolled barley and rolled triticale at 26% and 29% moisture increased N and P solubility and decreased ratio of phytate P to total P (Phytate P:Total P as a measure of phytate degradation) compared with grains before storage ($P<0.05$) and dry storage at d 49 ($P<0.05$). The added enzyme combination increased P solubility in all barley groups by 8-16% points ($P<0.001$) but only increased N solubility by 4% points in HMA stored rolled barley at 29% moisture at d 49 ($P<0.001$) compared with no enzyme addition. The enzyme combination also increased N (3% and 5% points on average) and P solubility (8% and 15% points) in HMA stored rolled triticale at 26% and 29% moisture, respectively ($P<0.05$). The inclusion of the enzyme combination during storage of rolled barley and rolled triticale for 49 days increased phytate degradation (23% and 39% points, respectively) and N (16% and 24% points, respectively) and P solubility (25% and 52% points, respectively) in HMA storage at 29% moisture to a greater extent compared with dry storage ($P<0.05$). At d 49, increasing moisture levels increased P solubility (rolled barley, whole and rolled triticale) and N solubility (whole and rolled triticale) linearly and decreased Phytate P:Total P (rolled barley) linearly. There was a positive linear correlation between P and N solubility of HMAS stored rolled barley and triticale. Overall, HMA storage of rolled barley and rolled triticale at 29% moisture with the enzyme combination is the suitable condition for increasing N and P solubility.

Keywords: Enzyme, Cereal, Phytase, Phytate, Pig, Protein

Abbreviations:
N, nitrogen; P, phosphorus; HMA, high moisture airtight; DM, dry matter; CP, crude protein; Phytate P:Total P, ratio between Phytate P and Total P; d, day; + E, with the enzyme combination; NSP, non-starch polysaccharides; ATTD, apparent total tract digestibility

1. Introduction

Cereals such as wheat, barley and triticale constitute up to 70% of a typical North European pig diet. However, cereal protein and phosphorus (P) are often embedded tightly in the grain matrix and are poorly digested by pigs due to the presence of anti-nutritional factors, for example non-starch polysaccharides (NSP) and phytate. In fact, NSP create a barrier preventing enzymes from accessing substrates bound within the cell wall (Baik and Ullrich, 2008), while phytate forms insoluble complexes with protein and P (Selle et al., 2000; Weremko et al., 1997). Therefore, unlocking the nutrient potential of cereal by different feed processes may improve protein and P digestibility and reduce N and P emissions to the environment.

Processing of cereals by means of grinding or rolling is used to rupture grains and render nutrients more accessible to digestive, plant and/or exogenous enzymes (Rowe et al., 1999). Moreover, using an enzyme combination (e.g. phytase, xylanase, β-glucanase and protease) may bring a potential synergistic effect on N and P digestibility. The supplementation of NSP degrading enzymes (xylanase and β-glucanase) together with phytase increases the accessibility of phytase to phytate (Adeola and Cowieson, 2011). Also, the addition of protease may support protein digestion. However, the effect on pigs is still unclear (Adeola and Cowieson, 2011). The activation of plant and exogenous enzymes requires moist conditions and is initiated in the stomach. Moreover, the time allowing enzymes’ active sites to access substrate is short, because N and P are mainly absorbed at the proximal part of the small intestine (Cross et al., 1990; Low, 1979; Partridge, 1978). Based on this perspective, high moisture airtight (HMA) storage can be a potential approach to improving nutrient availability as it creates ideal conditions for activating enzymes (high moisture) and for providing more time to degrade substrate (Choct and Hughes, 1997; Kim et al., 2005). In fact, HMA storage improved soluble protein in barley (Åman et al., 1990b) and maize (Baron et al., 1986) along with soluble P in maize (Niven et al., 2007). Nutrient solubility seems to be a sensitive marker of biological changes during HMA storage (Åman et al., 1990b). Phosphorus solubility is also used to estimate P availability (Columbus et al., 2010; Niven et al., 2007). Moreover, Christensen (2013a)
found the correlation between the increase of apparent total tract digestibility (ATTD) of barley protein and the ingested amount of soluble protein in pigs. Therefore, in the current study, N and P solubility was used as an indicator of the effect of HMA storage on improving nutrient availability of cereals. However, as mentioned above, the increase of nutrient solubility during HMA storage may depend on different factors such as moisture levels, storage time and grain processing. The reported effects varied (Âman et al., 1990b; Baron et al., 1986; Prigge et al., 1976) and are not yet well known. To our knowledge, no reports on exogenous enzyme addition during storage of cereal have been published.

The hypothesis of the current study is that HMA storage of barley and triticale activates endogenous enzymes prior to feeding, resulting in increased solubility of N and P compared with dry storage. Moreover, we hypothesise that together the addition of microbial phytase, xylanase, beta-glucanase and protease to barley during storage will increase the solubility of N and P compared with no enzyme addition. Therefore, the study aimed to evaluate the effect of storage time, grain processing (whole vs. rolled), enzyme combination and storage (dry storage vs. HMA storage at different moisture levels) on N and P solubility of barley and triticale.

2. Materials and methods

2.1. Cereals and experimental procedure

Barley and triticale (13% and 10% moisture, respectively) were cleaned separately using a sample cleaner MLN (Wintersteiger AG, Ried/I., Austria) to remove dust and unwanted particles. Half of the barley and triticale remained as whole grain (whole), whereas the other half was coarsely ground (rolled) by a roller mill (Skiold crushers KB 200, SKIOLD A/S, Sæby, Denmark). The average particle size of rolled barley and rolled triticale were 2560 ± 4 µm and 1960 ± 2 µm, respectively. Before storage, whole and rolled cereals were supplemented without or with an enzyme combination of phytase (Natuphos 5000G, BASF SE, Ludwigshafen, Germany) at 1000 FTU/kg of feed; xylanase (Danisco Xylanase 8000 G, Danisco Animal Nutrition, Marlborough, England) at 4000 U/kg of feed; β-glucanase (ECONASE Barley P700, AB Vista, Marlborough Wiltshire, United Kingdom) at 17500 BU/kg of feed and protease (RONOZYME ProAct CT, DSM Nutritional Products Ltd, Basel, Switzerland) at 15000 PROT/kg of feed.
Whole or rolled barley and triticale supplemented without or with the enzyme combination were either stored in 10 L covered plastic buckets (dry storage) at ambient temperatures (23°C on average) or in vacuum packed plastic bags providing airtight conditions for the cereals at moisture levels of 20, 23, 26 and 29% (HMA storage) (Table 1). Before HMA storage, the dry matter (DM) content of barley and triticale was determined. Based on the DM content, the required amounts of water were added to the barley and triticale to obtain the planned moisture levels. The procedure for HMA storage was as follows: each sample (400 g) of whole or rolled barley or triticale was weighed in a plastic bag. After mixing thoroughly with demineralised water, the bag was sealed by a vacuum packaging machine (Webomatic I 22-D, Bochum, Germany). The combination of phytase, xylanase, β-glucanase and protease was added to the enzyme supplemented groups at the same time as water. Samples in triplicate were randomly placed in an incubator (New Brunswick Scientific Model G25 Controlled Environment Incubator Shaker, GST Technical Sales, Edmonton, Alberta, Canada) at 15°C. The positions of samples in the incubator were changed randomly every week. The temperature of the incubator was measured every day at different positions (data not shown). The HMA storage samples were collected after 14, 29 and 49 days of storage, whereas dry stored samples were collected at d 0 and after 49 days of storage. After collection, samples were immediately frozen.

2.2. Chemical analysis

Samples were ground by a laboratory hammer mill with a 1 mm screen prior to analysis of DM, ash, crude protein (CP), total P and calcium (Ca), soluble N and soluble P. DM was determined by freeze drying. Total P was analysed by the method of Stuffins (1967) and Ca by atomic absorption spectrophotometry (model SP9, Pye Unicam Ltd., Cambridge, UK). For determination of soluble P and soluble nitrogen (N) analysis, a 30 g sample was mixed with 70 g of demineralised water on a magnetic stirrer at 550 rpm for 10 minutes, and the pH was measured (Radiometer, Copenhagen, Denmark). After centrifugation at 4100 rpm and at 4°C for 10 minutes, the supernatant was extracted and water soluble P was measured using the biuret method automated by Roche Hitachi 912 chemistry analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Soluble N in the supernatant and total N in feed was measured using Kjeltec AUTO 2400 Analyzer System (FOSS, Hillerød, Denmark) based on the Kjeldahl method 978.02 (AOAC, 1990). Moreover, samples were freeze-dried and milled using a 1 mm screen before analysing phytate P (Haug and Lantzsch, 1983), phytase activity (Engelen et al., 1994), xylanase activity
(measured at DuPont Nutrition Biosciences ApS (Brabrand, Denmark) using the colorimetric method and Xylazyme AX 60 mg from Megazyme (Wicklow, Ireland)) and β-glucanase (measured at Enzyme Services & Consultancy (ESC) Ltd. (Wales, UK) using Glycazyme tablets (60 mg) as a substrate (ESC Standard Analytical Method SAM043-01)).

2.3. Calculation

Crude protein (CP), N solubility, P solubility and ratio of phytate P to total P (Phytate P:Total P) as a measure of phytate degradation was calculated as follows:

\[ CP (g/kg DM) = \text{Total } N (g/kg DM) \times k \]

Where \(k\) is 5.45 for barley and 5.49 for triticale (Mariotti et al., 2008)

\[ N \text{ solubility} = \frac{\text{Soluble } N (g/kg DM)}{\text{Total } N (g/kg DM)} \]

\[ P \text{ solubility} = \frac{\text{Soluble } P (g/kg DM)}{\text{Total } P (g/kg DM)} \]

\[ \text{Phytate } P: \text{Total } P = \frac{\text{Phytate } P (g/kg DM)}{\text{Total } P (g/kg DM)} \]

2.4. Statistical analysis

All data were presented as least square mean and standard error of mean (n = 3). The data was analysed by applying general Gaussian (normal) linear models fit with the function lm() of the software R version 3.1.1 (R-Core-Team, 2014). The first model included the main effect of storage time (d 0, 14, 29 and 49), grain processing (whole vs. rolled), enzyme combination (without vs. with) and the interactions between main effects on N solubility and P solubility of barley or triticale separately at each moisture level (20%, 23%, 26% and 29%). The second model included the main effect of storage (dry storage, HMA storage at 20%, 23%, 26% and 29% moisture), grain processing (whole vs. rolled), enzyme combination (without vs. with) and the interactions between main effects on N solubility, P solubility, Phytate P:Total P, phytase activity and pH of each cereal separately at d 49. For each model used the presence of high order interactions (second and/or third order) were tested (standard F-test); interaction effects not statistically significant were eliminated. The models presenting significant third-order interactions were presented as separate analyses performed in suitably defined sub-sets of the data (Robinson et al., 2006). Some of the responses were found to follow a linear relationship with respect to the storage time or the moisture level; in that case the storage time or the moisture level was considered a continuous response variable (covariate) in the models, otherwise the storage time or the
moisture level entered in the model as a classification explanatory variable (factor).

Pairwise comparisons were performed by using the method of lsmeans implemented in
the R-package lsmeans (Lenth, 2014). The significance level of 5% was used through.

3. Results

On average over whole and rolled form, P solubility was similar for barley and triticale
(0.21 vs. 0.19, respectively, \( P=0.23 \)). However, triticale showed higher N solubility (0.31 vs.
0.17, \( P<0.001 \)). Phytate P:Total P (0.79 vs. 0.71, \( P=0.04 \)), phytase activity (1130 vs. 430
FTU/kg DM, \( P=0.004 \)), xylanase activity (200 vs. 100 U/kg DM, \( P<0.05 \)) and β-glucanase
activity (49400 vs. 5930 BU/kg DM, \( P<0.001 \)) than barley. The added enzyme activity was
not the same in barley and triticale as 740 vs. 1280 FTU/kg DM for phytase, respectively;
7580 vs. 6970 U/kg DM for xylanase, respectively; and 9570 vs. 8800 BU/kg DM for β-
1-glucanase, respectively.

3.1. N and P solubility of barley as affected by storage time, grain processing, enzyme
combination and storage method

HMA storage of whole or rolled barley at 20% moisture without or with the enzyme
combination did not increase N solubility at any time point compared with d 0 (\( P>0.05 \)). At
23% moisture level, N solubility increased by 1-2% point averaged over whole and rolled
grain and the enzyme combination levels at d 14, 29 and 49 compared with d 0 (\( P<0.001 \);
Table 2; however, rolling barley before storage decreased N solubility by 1% point
compared with whole barley (\( P<0.001 \); Fig. 1). In 26% moisture barley, N solubility was
affected by the interaction between storage time and grain processing (\( P=0.003 \)), as N
solubility only increased over time in rolled barley (Fig. 1). At 29% moisture, the three-way
interaction between storage time, grain processing and enzyme combination (\( P=0.002 \))
was observed because N solubility increased in rolled barley at all time points compared
with d 0 (\( P<0.05 \)), but only at d 49 in whole barley (\( P=0.04 \)) (Fig. 1). Moreover, at d 49,
enzyme combination enhanced N solubility of HMA stored rolled barley at 29% moisture by
4% points compared with no enzyme addition (\( P=0.025 \), Fig. 1).

The N solubility of barley at d 49 was affected by the three-way interaction between
storage, grain processing and enzyme combination (\( P=0.02 \); Table 2). This is because HMA
storage only enhanced N solubility of rolled barley at 26% moisture without the enzyme
combination (\( P=0.02 \)) and at 29% moisture without and with the enzyme combination.
(P<0.001) compared with dry storage (Table 2). The highest N solubility was observed in HMA stored 29% moisture rolled barley at d 49 (0.32 averaged over without and with enzymes addition, P<0.01).

At moisture levels above 20%, the three-way interaction of storage time, grain processing and enzyme combination influenced P solubility (P<0.001; Fig. 2). This was due to an increase over time of P solubility by HMA storage in whole barley with the enzyme combination and in rolled barley without or with the enzyme combination compared with d 0 (P<0.05), whereas HMA storage showed no effect or even decreased P solubility over time in whole barley without enzyme addition (P>0.05; Fig. 2). In fact, P solubility increased linearly over time in rolled barley at all moisture levels except at 26% moisture with the enzyme combination (Fig. 2). Adding the combination of phytase, xylanase, β-glucanase and protease increased P solubility by 8-16% points in whole and rolled barley at all moisture levels compared with no enzyme addition (P<0.05, Fig. 2). Table 2 shows the three-way interaction between storage, grain processing and enzyme combination on P solubility (P=0.004) and phytase activity (P<0.001) and their two-way interactions on Phytate P:Total P (P<0.05) in barley at d 49. This is because HMA storage enhanced P solubility of rolled barley at 26% and 29% moisture (without and with the enzyme combination) compared with dry storage and HMA storage at 20% moisture; however this positive effect was only observed in whole barley at above 20% moisture with the enzyme combination (P<0.05). For every additional percentage in moisture content of HMA stored rolled barley, P solubility would increase linearly by an average of 2% points (without the enzyme combination: Y = -0.20 + 1.99 x Moisture (%), R^2 = 0.96, P=0.54), and by 2.5% points when the enzyme combination was added (Y = -0.20 + 2.45 x Moisture (%), R^2 = 0.82, P=0.08). By increasing the moisture with 1% at d 49, Phytate P:Total P also decreased linearly by almost 2% points and by 3% points in HMA stored rolled barley without (Y = 1.10 - 1.62 x Moisture (%), R^2 = 0.81, P=0.08) or with the enzyme combination (Y = 1.31 - 2.70 x Moisture (%), R^2 = 0.87, P=0.15), respectively. A significant reduction of phytate P as shown in the decrease of Phytate P:Total P was found in rolled barley at 26% and 29% moisture in comparison with dry storage and other HMA stored groups (P<0.05; Table 2). The use of enzyme combination appeared to reduce Phytate P:Total P by 12% points in rolled barley at 29% moisture compared with no enzyme addition (P<0.01). Moreover, a higher phytase activity was observed in dry stored barley compared with HMA-stored barley at d 49.
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Besides, rolled barley exhibited higher phytase activity than whole barley at d 49 regardless of the added enzyme combination (P<0.01).

Averaged over without and with the enzyme combination at d 49, rolled barley had a lower pH than whole barley (5.8 vs. 6.0, P<0.001; Table 4). Also, pH of barley at 29% moisture (5.7) was lower than other HMA stored and dry stored treatments (5.9, P=0.04).

3.2. N and P solubility of triticale as affected by storage time, grain processing, enzyme combination and storage method

Above 20% moisture, the interaction effect between storage time and grain processing influenced N solubility of triticale (P<0.001; Fig. 3). This was due to an increase in N solubility over time in rolled triticale (P<0.05), but there was no effect on whole triticale (P>0.05). The difference between rolled and whole triticale was evident at d 14 and 49 at 23% moisture and at d 29 and 49 at 26% and 29% moisture (P<0.01; Fig. 3). Adding the enzyme combination enhanced N solubility of triticale at 26% moisture by 2% points compared with no enzyme addition, whereas the enhancement of N solubility by the enzyme combination was observed in triticale at 29% moisture at only d 14 (P<0.001; Fig. 3).

The two-way interaction of storage and grain processing was observed in N solubility of triticale at d 49 (P<0.001), because the increase of N solubility was only observed in HMA stored rolled barley at 26% and 29% moisture (P<0.05; Table 3). Adding the enzyme combination also increased N solubility of triticale to averaged 2% points compared with no enzyme addition (P=0.006; Table 3). Considering only the HMA storage groups at d 49, N solubility followed linear trends in whole triticale (without the enzyme combination: Y = 0.23 + 0.33 x Moisture (%)); with the enzyme combination: Y = 0.24 + 0.33 x Moisture (%), R² = 0.49, P=0.17) and rolled triticale (Y = - 0.09 + 2.04 x Moisture (%), R² = 0.80, P=0.09). Thus, increasing the moisture level by 1% in HMA stored triticale increased the N solubility by only 0.33% points in whole grain, but by 2% points in rolled grain.

High moisture airtight storage of triticale increased P solubility over time at all moisture levels compared with d 0 (P<0.05). At 26% moisture, the enzyme combination increased P solubility in rolled triticale at d 14 and 29 (8-9% points) and in whole triticale at d 14 and d 49 (2-3% points, P<0.05; Fig. 4). At 29% moisture, the enzyme combination increased P solubility at d 14 (11% points) and d 49 (14% points) compared with no enzyme addition.
and rolling triticale before HMA storage also enhanced P solubility at d 29 (31% points) and d 49 (24% points) compared with whole triticale ($P<0.001$; Fig. 4). The three-way interaction between storage method, grain processing and enzyme combination on P solubility ($P=0.01$). Phytate P:Total P ($P<0.001$) and phytase activity ($P<0.001$) of triticale at d 49 is shown in Table 5. This is because without the enzyme combination, HMA storage enhanced P solubility of whole and rolled triticale at moisture levels above 20% ($P<0.05$), whereas with the enzyme combination, P solubility was not increased in HMA stored whole barley at 23% moisture compared with dry stored barley ($P=0.109$; Table 3). Interestingly, P solubility increased linearly in HMA storage groups when increasing the moisture level in whole triticale (without the enzyme combination: $Y = 0.19 + 0.47 \times \text{Moisture (\%)}$, $R^2 = 0.79$, $P=0.13$; with the enzyme combination: $Y = -0.12 + 1.81 \times \text{Moisture (\%)}$) and in rolled triticale with the enzyme combination ($Y = -0.60 + 4.34 \times \text{Moisture (\%)}$, $R^2 = 0.90$, $P=0.1$). A percentage increase of moisture content in HMA stored triticale enhanced P solubility of whole grain by 0.5% (without the enzyme combination) or 2% (with the enzyme combination), but the increase was 4% points in rolled grain with the enzyme combination. Moreover, HMA storage reduced Phytate P:Total P in whole triticale at 29% moisture and rolled triticale at above 20% moisture compared with dry storage ($P<0.005$). The reduction of Phytate P:Total P in response to the added enzyme combination was observed in HMA stored rolled triticale at 23% moisture (7% points) and 29% moisture (17% points) at d 49 ($P<0.01$, Table 5). With the enzyme combination, 29% moisture triticale in rolled form had the highest P solubility ($P<0.001$) and the lowest ratio of Phytate P:Total P ($P<0.01$). Dry stored triticale had higher phytase activity compared with HMA stored triticale at d 49, except from rolled triticale at 20% and 23% moisture with the enzyme combination ($P<0.05$; Table 3). However, only HMA stored rolled triticale at 26% and 29% moisture with no enzymes, and at 29% moisture with enzyme combination had lower pH compared with dry stored whole and rolled triticale ($P<0.05$; Table 3). Considering only the rolled form at 29% moisture regardless of the enzyme combination, triticale had higher N (20% points, $P<0.001$) and P solubility (15% points, $P<0.001$) and lower phytate P (7% points, $P=0.003$) than barley at d 49.

### 3.3. Relationship between N and P solubility

HMA storage had limited or even no effect on N and P solubility of whole barley and whole triticale. Thus, no specific trend between N and P solubility was observed in whole barley and whole triticale. However, considering only rolled cereals after HMA storage,
there was a linear relationship between N and P solubility in barley and triticale (Fig. 5).

Using the combination of phytase, xylanase, β-glucanase and protease influenced this relationship in barley, but not in triticale, as N solubility was decreased by 6% points compared with no enzymes addition.

4. Discussion

The present results of N solubility in barley before storage are within the range (0.17 – 0.21) that was reported by Åman et al. (1990b). Triticale showed a higher N solubility than barley which corresponds with the higher protein solubility in triticale reported by Christensen (2013a). Moreover, P solubility of both barley and triticale is higher than previous result of maize (0.09) (Niven et al. (2007)). The observed differences between the planned and analyzed activity of the added enzymes in barley and triticale may be due to the differences in particle size and density rendering the collection of homogeneous samples problematic.

The effect of grain processing before storage on enhancing nutrient solubility of HMA stored barley was influenced by the moisture level of cereals in the current study. At moisture below 26%, the change of nutrient solubility due to HMA storage was limited in both barley and triticale, so grain processing had minor effect on nutrient solubility. In line with our results, Åman et al. (1990b) reported no effect of HMA storage on enhancing N solubility of whole barley at 23% and 25% moisture even after almost one year of storage. At 26% and 29% moisture, HMA storage of rolled barley and rolled triticale resulted in a higher solubility of N and P and a lower Phytate P:Total P compared with whole grains at d 49 in the current study. Likewise, HMA storage of ground maize at 26% and 33% moisture for 90 days enhanced N solubility to a greater extent (9% and 24% points, respectively) compared with whole maize (2 and 10% points, respectively) (Baron et al., 1986). According to Prigge et al. (1976), whole grains had a slower proteolysis rate which resulted in the limited N solubilisation compared with rolled grains. Moreover, grinding or rolling may disrupt the grain matrix and expose more substrate (N and P) for enzymatic and chemical attacks (Prigge et al., 1976).

The current study observed a positive linear correlation between moisture levels and P solubility (in barley and triticale) and N solubility (in triticale) at d 49. Accordingly, increasing moisture from 22% to 35% increased the rates and extent of proteolysis in maize (Baron et al., 1986). The enhancement of N solubility was only observed in HMA stored...
barley at 36% moisture, but not in barley at 23% and 25% moisture (Åman et al., 1990b). Increasing moisture levels in HMA stored barley and triticale linearly decreased Phytate P:Total P and at the same time increased P solubility. Abrams et al. (1976) observed a reduction of phytate P from 2.05 to 1.47 g/kg DM when ground maize was stored at 28% moisture for 21 days. Niven et al. (2007) found a higher P solubility (0.44) in maize at 25% moisture after 6 months of storage compared with the freshly harvested maize (0.09). The authors explained that the increase of soluble P mainly was due to the release of phytate-bound P during HMA storage which is also observed in the current study. Poulsen et al. (2012) observed a higher phytase activity in high-moisture stored barley and wheat (990 FTU/kg DM) in comparison with dry stored grains (870 FTU/kg DM). In contrast, the current study observed a higher phytase activity in dry stored groups and 20% moisture groups compared with higher moisture groups after 49 days of storage. Negative linear correlations were also found between phytase activity and moisture levels in HMA stored whole barley at d 49. This is most likely due to the fact that endogenous phytase was not activated in dry stored cereals and 20% moisture cereals. On the other hand, HMA storage at moisture levels above 20% activated endogenous phytase which was then diminished during storage. This result agreed with other studies that observed the disappearance of endogenous phytase after soaking for 24 h and 48 h (Carlson and Poulsen, 2003). The same trend may occur in other endogenous enzymes as the activity of endogenous β-glucanase in HMA stored barley was also decreased from 5900 BU/kg DM (before storage) to 3120 BU/kg DM at d 49 (Ton Nu, unpublished data). The increase of nutrient solubility after HMA storage is most likely because HMA storage activates endogenous enzymes and promotes proteolysis and fermentation process (Abrams et al., 1976; Åman et al., 1990b; Prigge et al., 1976). Furthermore, during HMA storage, the degradation of the starch-protein matrix made hydrophobic proteins more soluble and increased protein solubility in cereals (Hoffman et al., 2011). Microbial fermentation is associated with the production of organic acids (mostly lactic acid) which normally decreases the pH of HMA stored cereals below 5 (Baron et al., 1986; Buchanan-Smith et al., 2003). The current study only observed the drop of pH from 5.9 to 5.65 in barley, and from 6.70 to 5.74 in triticale after HMA storage. This indicates that the microbial fermentation was nil or very limited in barley and only a bit higher in triticale suggesting that the increase in N and P solubility mainly was due to the activity of endogenous enzymes in barley and triticale. Likewise, Prigge et al. (1976) also stated that microbial fermentation only had an indirect effect on promoting
proteolysis in HMA stored maize because N solubility was still increased after inhibiting fermentation.

The present positive linear relationship between storage time and P or N solubility of HMA stored rolled barley at 29% moisture indicated that a longer storage time resulted in higher P solubility. However, in general, prolonging HMA storage time had a limited effect on P solubility and N solubility in both barley and triticale, as the increase of P and N solubility could reach the plateau state after 14 or 29 days of storage. This is in accordance with previous results of HMA stored ground maize at 22%, 26% and 36% moisture where no difference in soluble N were found between 15, 30 or 90 days of storage (Baron et al., 1986). It means that the storage time can be flexible depending on practical conditions, and cereals can be kept in HMA conditions for a long period with no decrease of nutrient solubility.

It is important to note that the combination of phytase, xylanase, β-glucanase and protease increased P solubility in whole and rolled barley not only at d 14, 29 and 49, but also at day 0 regardless of storage. P solubility was measured from the supernatant after extracting with water for 10 minutes. Consequently, the enzymes might have been activated during the extraction process and thereby increased the P solubility. This is in agreement with previous findings which show that the improvement of nutrient solubility reflects reactions occurring not only during storage but also during the extraction process (Åman et al., 1990b). In fact, the current result was similar to the initial rapid phytate hydrolysis observed in previous studies with soaked wheat, soybean meal and rapeseed cake supplemented with microbial phytase (Blaabjerg et al., 2010a), maize (Niven et al., 2007) and rapeseed meal (Newkirk and Classen, 1998). These findings provide further evidence that one part of the phytate pool in plant feedstuffs is readily accessible for enzyme to degrade compared to other parts (Blaabjerg et al., 2010a; Blaabjerg et al., 2012). However, the degradation rate decreased due to the fact that phytate might form complexes with protein or minerals in the grain matrix, whereby phytate was inaccessible for phytase access (Newkirk and Classen, 1998). Interestingly, the increasing effect of the added enzyme combination on P solubility at d 0 was only observed in barley but not in triticale. The effect of the enzyme combination was consistent in triticale, as the increase in both N and P solubility was observed at the same time in rolled triticale at 26 and 29% moisture. Accordingly, the increase of P solubility in rolled triticale resulted in a
corresponding linear increase of N solubility, which was not influenced by the added enzyme combination. Except in 29% moisture rolled barley, the increase of P solubility in barley due to the addition of phytase, xylanase, β-glucanase and protease did not correspond with the increase of N solubility. The explanation could be differences in grain morphology between barley and triticale as well as substrate preference of the enzymes for P-compounds compared with N-compounds. According to Rodehutsscord et al. (1996) and Blaabjerg et al. (2010a), the high endogenous phytase activity in triticale may limit the response to exogenous phytase. In general, the addition of phytase, xylanase, β-glucanase and protease is more effective with regard to increasing the P solubility of barley than triticale.

The present results indicate that grain processing before storage (rolling) and moisture level are the main factors affecting N and P solubility of HMA stored barley and triticale. Rolled grains with moisture at 26% and 29% are required in HMA storage of barley and triticale to improve N and P solubility. However, the positive linear relationship between moisture content and the solubility of N and P indicates that moisture level is the limiting factor, and 29% moisture seems not to be the optimal condition to achieve the highest nutrient solubility. Therefore, a higher moisture content (>30%) may even lead to improvement of N and P solubility. As mentioned, N and P solubility was used in this study as indicators of N and P availability of cereals because water soluble inorganic P is readily for absorption in the small intestine (Ajakaiye et al., 2003) and soluble protein is more digestible than insoluble protein in vivo (Qiao et al., 2004b). Therefore, it is anticipated that the observed increase of N and P solubility after HMA storage also results in improvement of N and P digestibility of barley and triticale when fed to pigs. The effect on N and P digestibility of HMA stored cereals with moisture levels above 30% should be studied in pigs.

5. Conclusion

In general, high moisture airtight (HMA) storage of barley and triticale increased N and P solubility, even though the effect differed between whole or rolled grain, moisture level, storage time and enzyme combination. Processing of grain before storage by means of rolling and high moisture levels (26 and 29%) is required for the increase of N and P solubility and for the phytate degradation in HMA stored cereals. After 49 days, HMA storage of rolled barley and rolled triticale at 29% moisture increased N solubility (11 and
15% points, respectively) and P solubility (16 and 31% points, respectively) and decreased Phytate P:Total P (13 and 26% points, respectively) compared with dry stored cereals. The addition of phytase, xylanase, β-glucanase and protease increased the effect of HMA storage on rolled barley and rolled triticale at 29% moisture even more by a higher increase of N (4 and 7% points respectively) and P solubility (16 and 18% points respectively) and by a greater reduction of Phytate P:Total P (12 and 17% points respectively) compared with no enzyme addition. Also, the increase of P solubility in HMA stored rolled barley and triticale was positively correlated to N solubility. Moreover, increasing the moisture level of HMA stored barley and triticale from 20 to 29% led to a linear increase of P (in rolled barley; whole and rolled triticale) and N solubility (whole and rolled triticale) and to a linear decrease in Phytate P:Total P (in rolled barley). These results strongly indicate that moisture levels above 30% may result in an even higher increase of nutrient solubility.

Conflict of interest
The authors declare that there are no conflicts of interest.

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Reference


Fig. 1. Effect of storage time (T: 0, 14, 29 and 49 days), grain processing (G: whole vs. rolled), enzyme combination (E: without vs. with phytase, xylanase, β-glucanase and protease (+E)) on N solubility after high moisture airtight (HMA) storage of barley at: (A) 20% moisture (T x G x E, \(P=0.022\)): whole (T, \(P<0.001\); E, \(P=0.306\); T x E, \(P=0.090\)); rolled (T x E, \(P=0.021\)); (B) 23% moisture (T, \(P<0.001\); G, \(P<0.001\); E, \(P=0.171\)); (C) 26% moisture (T x G, \(P=0.005\); T x E, \(P=0.547\); G x E, \(P=0.625\)); \(a-d\) Mean values of whole barley, \(a-d\) Mean values of rolled barley; (D) 29% moisture (T x G x E, \(P=0.002\)): whole (T, \(P=0.031\); E, \(P=0.813\); T x E, \(P=0.306\)); rolled: (T x E, \(P=0.006\). A linear relationship was observed: without enzyme (whole: \(Y = 0.186 + 0.0002 \times \text{Storage time (day)}\); rolled: \(Y = 0.17 + 0.003 \times \text{Storage time (day)}, R^2 = 0.92, P = 0.06\)); \(a-d\) Mean values of rolled barley without enzyme; \(a-d\) Mean values of rolled barley with enzyme; \(A-B\) Mean value at each time point; \(a-d, A-B\) Mean values in each graph with unlike letters (regardless of bold or unbold) were different (\(P<0.05\)).
Fig. 2. Effect of storage time (T: 0, 14, 29 and 49 days), grain processing (G: whole vs. rolled), enzyme combination (E: without vs. with phytase, xylanase, β-glucanase and protease (+E)) on N solubility after high moisture airtight (HMA) storage of barley at: (A) 20% moisture (T x G, P<0.001; T x E, P<0.001; G x E, P=0.002): whole (T x E, P<0.001), rolled (T, P=0.039; E, P<0.001; T x E, P=0.143); (B) 23% moisture (T x G x E, P<0.001): whole (T x E, P<0.001), rolled (T x E, P<0.001); (C) 26% moisture (T x G x E, P<0.001): whole (T x E, P<0.001), rolled (T, P<0.001; E, P<0.001; T x E, P=0.120); (D) 29% moisture (T x G x E, P<0.001): whole (T x E, P<0.01).
rolled (T x E, $P=0.029$). A linear relationship was observed in rolled barley at 20% moisture (without enzymes: $Y = 0.185 + 0.0004 \times Storage\ time\ (day)$, with enzymes: $Y = 0.287 + 0.0004 \times Storage\ time\ (day)$, $R^2 = 0.95$, $P=0.34$). 23% moisture (without enzymes: $Y = 0.188 + 0.001 \times Storage\ time\ (day)$, with enzymes: $Y = 0.28 + 0.002 \times Storage\ time\ (day)$, $R^2 = 0.95$, $P=0.34$), 26% moisture (without enzymes: $Y = 0.198 + 0.002 \times Storage\ time\ (day)$, $R^2 = 0.81$, $P=0.12$) and 29% moisture (without enzymes: $Y = 0.205 + 0.004 \times Storage\ time\ (day)$, with enzymes: $Y = 0.293 + 0.005 \times Storage\ time\ (day)$, $R^2 = 0.95$, $P=0.07$).
Fig. 3. Effect of storage time (T: 0, 14, 29 and 49 days), grain processing (G: whole vs. rolled), enzyme combination (E: without vs. with phytase, xylanase, β-glucanase and protease (+E)) on N solubility after high moisture airtight (HMA) storage of triticale at: (A) 20% moisture (T, \( P=0.002; \) G, \( P<0.001; \) E, \( P=0.111 \)), (B) 23% moisture (T x G, \( P=0.001; \) T x E, \( P=0.313; \) G x E, \( P=0.233 \)); (C) 26% moisture (T x G, \( P<0.001; \) E, \( P<0.001 \)); (D) 29% moisture (T x G, \( P<0.001; \) T x E, \( P=0.028; \) G x E, \( P=0.395 \)); a-d Mean values of whole barley; a-d Mean values of rolled barley; A-B Mean value at each time point; a-c A-B Mean values in each graph with unlike letters (regardless of bold or unbold) were different (\( P<0.05 \)); x-y Significant difference of N solubility of HMA stored barley at 29% moisture without vs. with enzyme at d 14.
Fig. 4. Effect of storage time (T: 0, 14, 29 and 49 days), grain processing (G: whole vs. rolled), enzyme combination (E: without vs. with phytase, xylanase, β-glucanase and protease (+E)) on N solubility after high moisture airtight (HMA) storage of triticale at: (A) 20% moisture (T x G x E, P=0.023): whole (T, P<0.001; E, P=0.862; T x E, P=0.301), rolled (T, P<0.001; E, P=0.414; T x E, P=0.110); (B) 23% moisture (T x G x E, P=0.04): whole (T, P<0.01; E, P=0.003; T x E, P=0.338); (C) 26% moisture (T x G x E, P<0.001): whole (T x E, P<0.001), rolled (T x E, P<0.001). a-d Mean value of whole
or rolled triticale without enzymes. $^{a-d}$ Mean value of whole or rolled triticale with enzyme;

(D) 29% moisture (T x G, $P<0.001$; T x E, $P<0.001$; G x E, $P=0.213$): $^{a-d}$ Mean value of whole triticale. $^{a-d}$ Mean value of rolled triticale. $^{x-y}$ Significant difference of N solubility of HMA stored barley at 29% moisture without vs. with enzyme at d 14 and d 49. $^{A-C}$ Mean value at each time point; $^{a-d}$, $^{A-C}$ Mean values in each graph with unlike letters (regardless of bold or unbold) were different ($P<0.05$).
Fig. 5. Linear relationship between P solubility and N solubility in rolled barley (a) and rolled triticale (b) after high moisture airtight (HMA) storage from 0 to 49 days. In barley: without the enzyme combination: $N_{\text{solubility}} = 0.059 + 0.555 \times P_{\text{solubility}}$; with the enzyme combination (phytase, xylanase, $\beta$-glucanase and protease): $N_{\text{solubility}} = 0.002 + 0.555 \times P_{\text{solubility}}$ ($R^2 = 0.86$). In triticale: $N_{\text{solubility}} = 0.224 + 0.431 \times P_{\text{solubility}}$ ($R^2 = 0.88$).
Table 1
Experimental design.

<table>
<thead>
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<th>Storage</th>
<th>Cereal type</th>
<th>Moisture (%)</th>
<th>Grain processing</th>
<th>Enzyme combination&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Storage time (day)</th>
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<td>13</td>
<td>Whole grain</td>
<td>-/+</td>
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<td>0 and 49</td>
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<tr>
<td>HMA storage&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Barley</td>
<td>20, 23, 26 and 29</td>
<td>Whole grain</td>
<td>-/+</td>
<td>0, 14, 29 and 49</td>
</tr>
<tr>
<td></td>
<td>Triticale</td>
<td>20, 23, 26 and 29</td>
<td>Whole grain</td>
<td>-/+</td>
<td>0, 14, 29 and 49</td>
</tr>
</tbody>
</table>

<sup>a</sup>-/+: without or with the added enzyme combination of phytase (1000 FTU/kg of feed), xylanase (4000 U/kg of feed), β-glucanase (17500 BU/kg of feed) and protease (15000 PROT/kg of feed).

<sup>b</sup>HMA storage = high moisture airtight storage at 15°C.
Table 2

Effect of storage (dry storage vs. high moisture airtight (HMA) storage), grain processing and enzyme combination on N solubility, P solubility, Phytate P:Total P, phytase activity and pH of barley after 49 days.

<table>
<thead>
<tr>
<th>Processing</th>
<th>Enzyme(^a) Storage(^b)</th>
<th>N solubility</th>
<th>P solubility(^d)</th>
<th>Phytate P:Total P</th>
<th>Phytase activity(^d) (FTU/kg DM)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole barley</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry storage</td>
<td></td>
<td>0.17(^AB)</td>
<td>0.19(^A)</td>
<td>0.22(^AC)</td>
<td>0.31(^A)</td>
<td>0.75(^A)</td>
</tr>
<tr>
<td>HMA storage</td>
<td></td>
<td>0.16(^A)</td>
<td>0.17(^B)</td>
<td>0.17(^B)</td>
<td>0.30(^A)</td>
<td>0.75(^A)</td>
</tr>
<tr>
<td>20%</td>
<td></td>
<td>0.19(^AB)</td>
<td>0.20(^A)</td>
<td>0.18(^B)</td>
<td>0.41(^B)</td>
<td>0.76(^A)</td>
</tr>
<tr>
<td>23%</td>
<td></td>
<td>0.20(^ABC)</td>
<td>0.20(^A)</td>
<td>0.17(^B)</td>
<td>0.42(^B)</td>
<td>0.79(^A)</td>
</tr>
<tr>
<td>26%</td>
<td></td>
<td>0.20(^AB)</td>
<td>0.20(^A)</td>
<td>0.21(^AB)</td>
<td>0.41(^B)</td>
<td>0.76(^A)</td>
</tr>
<tr>
<td>29%</td>
<td></td>
<td>0.20(^B)</td>
<td>0.20(^A)</td>
<td>0.20(^A)</td>
<td>0.31(^A)</td>
<td>0.67(^B)</td>
</tr>
<tr>
<td>Rolled barley</td>
<td></td>
<td>0.19(^AB)</td>
<td>0.18(^A)</td>
<td>0.22(^AC)</td>
<td>0.29(^A)</td>
<td>0.76(^A)</td>
</tr>
<tr>
<td>HMA storage</td>
<td></td>
<td>0.17(^AB)</td>
<td>0.18(^A)</td>
<td>0.20(^A)</td>
<td>0.31(^A)</td>
<td>0.76(^A)</td>
</tr>
<tr>
<td>20%</td>
<td></td>
<td>0.18(^AB)</td>
<td>0.17(^A)</td>
<td>0.25(^C)</td>
<td>0.38(^AB)</td>
<td>0.76(^A)</td>
</tr>
<tr>
<td>23%</td>
<td></td>
<td>0.22(^C)</td>
<td>0.20(^A)</td>
<td>0.31(^D)</td>
<td>0.40(^B)</td>
<td>0.67(^B)</td>
</tr>
<tr>
<td>26%</td>
<td></td>
<td>0.30(^D)</td>
<td>0.34(^E)</td>
<td>0.38(^C)</td>
<td>0.54(^C)</td>
<td>0.63(^BX)</td>
</tr>
</tbody>
</table>

SEM  0.008  0.013  0.013  90  0.05

P-value\(^c\)

- Storage (S)  <0.001
- Processing (G)  <0.001
- Enzyme (E)  0.260
- S x E  0.020  0.224
- S x G  <0.001  0.050
- G x E  <0.001  0.411
- S x G x E  0.019  <0.001  0.142  <0.001  0.065

\( ^a \) /+ = without or with the enzyme combination (phytase, xylanase, β-glucanase and protease).

\( ^b \) Cereal was stored dry (dry storage) or under airtight conditions (HMA storage) at different moisture levels (20%, 23%, 26% and 29%).
S = storage method (dry storage vs. HMA storage 20% moisture vs. HMA storage 23% moisture vs. HMA storage 26% moisture vs. HMA storage 29% moisture), G = grain processing (whole grain vs. rolled grain), E = enzyme combination (without vs. with enzyme combination); S x G x E = the interaction between storage method, grain processing and enzyme combination; S x E = the interaction between storage method and enzyme combination; S x G = the interaction between storage method and grain processing; G x E = the interaction between grain processing and enzyme combination.

A-E Means within columns followed by different letters are different (P<0.05).

X-Y Means within rows within the same parameter followed by the different letters are different (P<0.05)

d-X/Y = All means within rows of P solubility and phytase activity are different (P<0.05).
<table>
<thead>
<tr>
<th>Processing</th>
<th>Enzyme&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Storage&lt;sup&gt;b&lt;/sup&gt;</th>
<th>N solubility</th>
<th>P solubility</th>
<th>Phytate P:Total P</th>
<th>Phytase activity&lt;sup&gt;c&lt;/sup&gt; (FTU/kg DM)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole triticale</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry storage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.33</td>
<td>0.35</td>
<td>0.34&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.20&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.21&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.80&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.82&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1232&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>HMA storage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>0.31</td>
<td>0.30&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.26&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.25&lt;sup&gt;ABC&lt;/sup&gt;</td>
<td>0.82&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.80&lt;sup&gt;A&lt;/sup&gt;</td>
<td>657&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.29</td>
<td>0.30</td>
<td>0.30&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.26&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.29&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>0.80&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.81&lt;sup&gt;A&lt;/sup&gt;</td>
<td>277&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.31</td>
<td>0.33</td>
<td>0.32&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.30&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>0.33&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>0.79&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.76&lt;sup&gt;A&lt;/sup&gt;</td>
<td>175&lt;sup&gt;CD&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.32</td>
<td>0.33</td>
<td>0.33&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;CX&lt;/sup&gt;</td>
<td>0.42&lt;sup&gt;EX&lt;/sup&gt;</td>
<td>0.71&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.67&lt;sup&gt;B&lt;/sup&gt;</td>
<td>84&lt;sup&gt;BD&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rolled triticale</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry storage</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0.33</td>
<td>0.31</td>
<td>0.32&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.21&lt;sup&gt;AD&lt;/sup&gt;</td>
<td>0.18&lt;sup&gt;A7&lt;/sup&gt;</td>
<td>0.84&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.80&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1189&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>HMA storage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.33</td>
<td>0.34</td>
<td>0.34&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.26&lt;sup&gt;BD&lt;/sup&gt;</td>
<td>0.28&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>0.77&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.79&lt;sup&gt;A&lt;/sup&gt;</td>
<td>905&lt;sup&gt;E&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.36</td>
<td>0.37</td>
<td>0.36&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.34&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>0.38&lt;sup&gt;DE&lt;/sup&gt;</td>
<td>0.73&lt;sup&gt;BX&lt;/sup&gt;</td>
<td>0.66&lt;sup&gt;BY&lt;/sup&gt;</td>
<td>558&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.40</td>
<td>0.44</td>
<td>0.42&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.51&lt;sup&gt;E&lt;/sup&gt;</td>
<td>0.47&lt;sup&gt;E&lt;/sup&gt;</td>
<td>0.65&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0.62&lt;sup&gt;B&lt;/sup&gt;</td>
<td>393&lt;sup&gt;F&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.48</td>
<td>0.55</td>
<td>0.52&lt;sup&gt;D&lt;/sup&gt;</td>
<td>0.52&lt;sup&gt;EX&lt;/sup&gt;</td>
<td>0.70&lt;sup&gt;FY&lt;/sup&gt;</td>
<td>0.58&lt;sup&gt;DX&lt;/sup&gt;</td>
<td>0.41&lt;sup&gt;CY&lt;/sup&gt;</td>
<td>195&lt;sup&gt;CD&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM</td>
<td>0.013</td>
<td>0.015</td>
<td>0.013</td>
<td>32</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>-/+ = without or with the enzyme combination (phytase, xylanase, β-glucanase and protease).

<sup>b</sup>Cereal was stored dry (dry storage) or under airtight conditions (HMA storage) at different moisture levels (20%, 23%, 26% and 29%).

<sup>c</sup>P-value<sup>c</sup>

| Storage (S) | 0.006 |
| Grain processing (G) | 0.154 |
| Enzyme (E) | <0.001 |
| S x E | 0.416 |
| S x G | 0.152 |
| G x E | 0.014 |
| S x G x E | <0.001 | <0.001 | 0.016 |
S = storage method (dry storage vs. HMA storage 20% moisture vs. HMA storage 23% moisture vs. HMA storage 26% moisture vs. HMA storage 29% moisture), G = grain processing (whole grain vs. rolled grain), E = enzyme combination (without vs. with enzyme combination); S x P x E = the interaction between storage method, grain processing and enzyme combination; S x E = the interaction between storage method and enzyme combination; S x G = the interaction between storage method and grain processing; G x E = the interaction between grain processing and enzyme combination.

Means (averaged over without and with enzymes) showed the interaction between storage method and grain processing (S x G). Means within columns followed by different letters are different (P<0.05). Means within rows and parameters followed by the different letters are different (P<0.05). All means within rows of phytase activity are different (P<0.05).
Manuscript II

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

M. A. Ton Nu, K. Blaabjerg, and H.D. Poulsen

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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 as the optimum moisture level for HMA storage of barley because it enhanced the phytate degradation (16% points) and the solubility of P (31% points), nitrogen (N) (20% points) and protein (9% points) compared to dry storage ($P < 0.05$) and at the same time may prevent a loss of energy and N via ammonia by microbial fermentation. In Exp. 2, 6 female growing pigs ($43 \pm 2$ kg) were used per diet (8 diets, 48 pigs) in a 12-d nutrient balance study to assess the effect of HMA storage of barley at 35% moisture and enzyme combination on the apparent total tract digestibility (ATTD) of P in barley fed alone or together with soybean meal (SBM). Alos, HMA storage of barley at 35% moisture without and with enzyme combination enhanced phytate degradation (averaged 26% points) and P solubility (averaged 53% points) ($P < 0.05$). Thus, HMA storage of barley (solely endogenous enzymes) enhanced ATTD of P to the same extent as dry stored barley with enzyme combination (endogenous enzymes plus added enzymes) in the barley diet (averaged 47%) and the barley-SBM diet (averaged 15%) ($P < 0.05$). Adding an enzyme combination to HMA stored barley achieved the greatest increase of ATTD of P in the barley diet (70%) and the barley-SBM diet (36%) ($P < 0.05$). The greater ATTD of P by HMA storage and the enzyme combination increased the urinary P excretion in barley and barley-SBM diets, as the dietary Ca supply was insufficient to support an increase in P retention ($P < 0.05$). The inclusion of the enzyme combination to HMA stored barley tended to enhance the calculated ATTD of P of SBM (13% points) compared with no enzyme combination ($P = 0.06$) due to the lower endogenous enzymes activity in HMA stored barley. Thus, it is necessary to add the enzyme combination to enhance the ATTD of P of SBM when fed together with HMA stored barley. Overall, HMA storage with enzyme combination is a potential method to enhance ATTD of P of barley in pigs to reduce the dietary need for inorganic P addition and P excretion to the environment.

**Key words:** barley, enzyme, high moisture airtight storage, phosphorus digestibility, phytate, pig

**INTRODUCTION**
Environmental concern related to excessive P and nitrogen (N) excretion is a challenge to modern pig production. The high P and N excretion is often associated with low digestibility of P and N of cereals, although cereals often contain a lot of P, protein and AA contributing to meet the pigs’ requirement. Non-starch polysaccharides (NSP) and phytate tightly embed protein and bind e.g. P which may hinder an increased digestibility of cereal nutrients (Eeckhout and Depaepe, 1994; Fincher, 1975). Microbial phytase degrades phytate and enhances apparent total tract digestibility (ATTD) of P up to 65% (Blaabjerg et al., 2012; Poulsen et al., 2007) whereas reported effects on apparent ileal digestibility (AID) of N in pigs are inconsistent (Selle and Ravindran, 2008). High moisture airtight (HMA) storage of barley may be a promising approach to release P, protein, and AA for absorption because the moist conditions in HMA storage activate plant and microbial enzymes to degrade NSP and phytate. Thus, HMA storage enhances soluble P (Niven et al., 2007) and phytate degradation in corn (Abrams et al., 1976) and soluble N in barley (Åman et al., 1990). Adding a combination of phytase, xylanase, β-glucanase and protease during HMA storage (up to 30% moisture) increased nutrient solubility in barley but apparently without reaching the maximum increase in P and N solubility (M.A. Ton Nu, unpublished data). Thus, we hypothesize that HMA storage of barley above 30% moisture with the added enzyme combination enhances P and N solubility which will increase the ATTD of P in HMA stored compared to dry stored barley when fed alone or together with soybean meal (SBM). The aim is as follows: (1) to define the optimal moisture level for maximum P and N solubility in HMA stored 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 ATTD and balance of P in pigs fed barley alone or together with SBM.

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 ATTD and balance of P in pigs fed a barley diet or a barley-SBM diet.

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 (Skiodl crushers KB 200, SKIOLD A/S, Sæby, Denmark) and divided
into two parts for dry storage or HMA storage. Coarsely rolled barley had an average particle size of 2,074 ± 1.83 µm. Before storing, rolled barley was added without or with a combination of phytase (Natuphos 5000G, BASF SE, Ludwigshafen, Germany) at 1,000 FTU/kg of feed; xylanase (Danisco Xylanase 8000 G, Danisco Animal Nutrition, Marlborough, England) at 4,000 U/kg of feed; β-glucanase (ECONASE Barley P700, AB Vista, Marlborough Wiltshire, United Kingdom) at 17,500 BU/kg of feed; and protease (RONOZYME ProAct CT, DSM Nutritional Products Ltd, Basel, Switzerland) at 15,000 PROT/kg of feed.

**Experiment 1**

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 according to M.A. Ton Nu (unpublished data). Samples in triplicate 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 calcium (Ca), soluble N, soluble protein, soluble P, phytate P, and enzyme activity (phytase, xylanase and β-glucanase).

**Experiment 2**

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

**Animals**

Forty-eight female pigs (43 ± 2 kg) were assigned randomly to the barley diet or to the barley-SBM diet in a 2 x 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 x 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: 5 d for adaptation, 7 d for total collection of urine and feces. On day 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 of a 30% acid sulfuric (H₂SO₄) addition to diminish ammonia evaporation. Feces collection 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.

**Preparation of feedstuffs and diets**

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 then immediately frozen for further analysis. Samples were processed as described in Exp. 1 prior to the analysis of pH, ash, total P and calcium (Ca), soluble P, phytate P, and enzyme activity (phytase, and β-glucanase).

Dry stored barley and HMA stored barley were reground by a hammer mill (3.5-mm screen size). Based on the Danish recommendation for all nutrients except protein, P and Ca (Pig Research Centre, 2014), the experimental diets were prepared according to Table 3. No crystalline amino acids and mineral phosphate were added. The dry stored barley was divided into 2 parts and mixed without or with SBM (30%, Table 4). 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.

**Sample analysis**

DM was determined by oven drying at 103°C for 20 hours. 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 3h in step 1 and for 1h in step 2. Cl 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). Samples were analyzed for phytase activity as described by Engelen et al. (1994). β-glucanase activity was
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 × g for 10 minutes, and pH was measured (Radiometer, Copenhagen, Denmark). The supernatant was extracted after being centrifuged at 2000 × g and 4°C for 10 minutes, 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 which can quantify tripeptides and larger polypeptides or protein, but cannot detect 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 NPN. Na content in diets was determined by the atomic

**Calculation**

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:

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

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)

Apparent total tract digestibility of P in SBM (%) was calculated by the difference from the barley-SBM diets and the respective barley diets as following:

\[
\text{ATTD of P in SBM} (%) = \left( \frac{P_{\text{barley-SBM}} \times \text{DigP}_{\text{barley-SBM}} - a \times P_{\text{barley}} \times \text{DigP}_{\text{barley}}}{b \times P_{\text{SBM}}} \right)
\]

Where: \( P_{\text{barley-SBM}} = \) total P of the barley-SBM diet (g/kg DM); \( \text{DigP}_{\text{barley-SBM}} = \) the determined ATTD of P of barley-SBM diet (%); \( a = \) proportion of barley in the barley-SBM diet; \( P_{\text{barley}} = \) total P of the respective barley (g/kg DM); \( \text{DigP}_{\text{barley}} = \) the determined ATTD of P in the barley diet (%); \( b = \) proportion of SBM in the barley-SBM diet; \( P_{\text{SBM}} = \) total P of SBM (g/kg DM).
Data were analyzed by use of 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 are 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 achieved in samples 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 for the barley diet. The same procedure was used to analyse the results of the barley-SBM diets as well as on the calculated ATTD of P in SBM. The pig was the experimental unit for all analyses. Treatment differences were separated by the PDIF option of SAS software.

**RESULTS**

**Experiment 1- small scale study**

The added phytase activity in barley at d 0 (1,500 FTU/kg DM) and in dry stored barley at d 49 (1200 FTU/kg DM) was greater than planned (1,000 FTU/kg diet). The analyzed enzyme activity in barley without or with the enzyme combination at d 0 was below 100 and 2,500 U/kg DM for xylanase, respectively and 5,900 and 20,000 U/kg DM for β-glucanase, respectively. Thus, the added xylanase (2,400 U/kg DM) and β-glucanase activity (14,100 U/kg DM) in barley were lower than planned (4,000 and 17,800 U/kg diet).

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). 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 (16% and 31% points, respectively), and the solubility of P (31% and 56% points, respectively), N (20% and 36% points, respectively) and protein (9% and 17% points, respectively) in barley at 35% and 40% moisture compared with dry storage ($P < 0.001$), whereas phytate degradation and nutrient solubility were not increased in HMA stored barley at 29% moisture ($P > 0.05$). Moreover, the enzyme combination enhanced P solubility by 13% to 30% points in HMA stored barley irrespective
of 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 during storage also improved the solubility of N (by 10% points) and protein (by 7% points) in HMA stored barley at 40% moisture. The effect of the enzyme combination on phytate degradation was only observed in barley stored at 35% and 40% moisture (by 22% and 21% points, respectively, $P < 0.001$). 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 of HMA stored barley was lower than dry stored barley ($P < 0.001$).

**Experiment 2**

The added phytase (1,500 FTU/kg DM) and xylanase (3,600 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 (1,270 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 53% points compared with dry storage ($P < 0.001$, Table 2). The added enzyme combination increased the P solubility of barley by 11% points 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 the detection level (< 50 FTU/kg DM). The diet composition and analyzed content are given in Table 3. Averaged over the levels of enzyme addition, barley-SBM diets had greater total P (4.5 vs. 3.3 g/kg DM) and Ca (1.2 vs.
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0.5 g/kg DM) than the 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.

Barley diet

In 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 ATTD of DM in pigs ($P > 0.05$; Table 4), whereas HMA storage increased ATTD of P of barley by 15% unit ($P < 0.001$). At the same time, P excretion in urine was increased by 0.40 g/d ($P = 0.005$) in pigs fed HMA stored barley. Inclusion of the enzyme combination during the storage of barley increased ATTD of P by 13% unit and P excretion in urine by 0.52 g/d ($P < 0.001$).

The interaction between storage and enzyme combination influenced P absorption and Ca retention ($P = 0.011$; Table 4), because pigs fed the HMA stored barley had a greater daily P absorption (0.82 g) and greater Ca retention (0.45 g) compared with pigs fed dry stored barley when no enzymes were added ($P < 0.001$), whereas no differences were observed when the enzyme combination was added ($P < 0.05$) (Table 4). The content of digestible P was 0.68 g/kg DM greater in HMA stored barley without the enzyme combination ($P < 0.001$) and 0.38 g/kg DM with the enzyme combination ($P = 0.013$) compared to dry stored barley.

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). However, 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 47 g/d than pigs fed HMA stored barley-SBM diet ($P = 0.002$), but no difference was observed when enzyme combination was added ($P = 0.66$) (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 ATTD of P of the barley-SBM diet by 6% unit ($P < 0.001$) 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 0.22
g greater in HMA stored barley-SBM diets compared with dry stored barley-SBM diets \( (P = 0.003, \text{Table 5}) \).

The addition of phytase, xylanase, beta-glucanase, and protease during storage increased ATTD of P (by 11% unit) and digestible P (by 0.45 g/kg DM) compared with no enzyme addition \( (P < 0.001, \text{Table 5}) \). However, no effect of storage or enzyme combination on the retention of Ca and P were observed \( (P > 0.05, \text{Table 5}) \). In general, the Ca intake was very low and limiting for the retention of both Ca and P (Table 5).

The calculated ATTD of P of SBM was based on the obtained results of barley (dry stored vs. HMA stored) without or with the enzyme combination and assuming the additivity of ATTD of P of the feedstuffs (Table 6). The ATTD of P of SBM fed together with dry stored barley was calculated to be 7% points greater compared with SBM fed together with HMA stored barley \( (P = 0.043) \). Furthermore, mixing SBM and HMA barley stored with the enzyme combination tended to increase ATTD of P of SBM by 13% points compared with no enzyme addition \( (P = 0.06) \).

**DISCUSSION**

Differences in particle size and density may result in difficulties in taking homogeneous samples from the mixture of rolled grains and added enzymes. This is probably the explanation for the deviation between the analyzed activity and the planned levels in both experiments.

**Experiment 1**

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 current protein solubility of HMA stored barley at 35% and 40% moisture was similar to the observed results of barley soaked with acetic acid or lactic acid for 12 hours (0.25 – 0.28) and 48 hours (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 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 to 6) after storage at moisture levels up to 35% indicated 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 lower Phytate P:Total P and nutrient solubility in HMA stored barley at 29% moisture compared with storage at 35% and 40% moisture. Carlson and Poulsen (2003) observed that soaking barley at 20°C led to a faster degradation rate of phytate compared with that at 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 to 18°C (M. A. Ton Nu, unpublished data). Both studies comprised the cultivar (Zephyr), and 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) (M. A. Ton Nu, unpublished data). 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 of storage scale (lab scale (400 g) vs. barn scale (400 kg) and storage conditions (in 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.

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 previous findings showing that the addition of exogenous enzymes to HMA stored barley is more effective in enhancing P than N solubility (M. A. Ton Nu, unpublished data).

In the current study, HMA storage of barley at 40% moisture achieved the lowest (negligible) amounts of phytate and the greatest nutrient solubility. Svihus et al. (1997) observed that HMA storage of barley at 40% moisture reduced protein (14% to 32%) and AA contents (especially Arg and Lys) and increased ammonia loss (8% to 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 (Humer et al., 2013). High moisture airtight stored triticale or wheat at 35% moisture accompanied by a high lactic acid production that dropped the pH to 3.9 consequently failed to improve ATTD of P in pigs (Pieper et al., 2011). Thus, 35% moisture was chosen for Exp. 2 to prevent the latent loss of energy and N via ammonia by microbial fermentation which was nil or very limited during HMA storage of barley at 35% moisture.

**Experiment 2**

The current effect of HMA storage on enhancing ATTD of P in barley was also observed in triticale and wheat at 25% moisture (32% and 22% points, respectively) (Pieper et al., 2011), and corn at 23% and 25% moisture (14% and 9% points) (Humer et al., 2013). Additionally, AID of P increased from 23% to 38% in HMA stored barley (29% moisture) compared with dry barley (Weltzien and Aherne, 1987). Svihus et al. (1997) also observed an increase of AID of P in broilers fed HMA stored barley, oat, or wheat at 40% moisture (19%, 11%, 9% points, respectively).

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 ATTD of P of barley due to HMA storage and enzyme combination correlated positively with the increase in P solubility.

High moisture airtight storage enhanced ATTD 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 ATTD of P was enhanced by 8% points in the mixed corn-barley-
SBM diet (Humer et al., 2014) but 14% points 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 digestive tract – in average 5 to 6 h –
becomes the limiting factor that affects the efficacy of both endogenous enzymes in
barley and added microbial enzymes to improve ATTD of P. Thus, in the current study, the
addition of the enzyme combination to dry stored barley (endogenous enzymes plus
added enzymes) enhanced ATTD of P to the same extent as HMA storage of barley at 35%
motion 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 ATTD 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 (< 1g/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, are tightly regulated together (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 metabolic Ca and P for bone mineralization are imbalanced,
increasing digestible P of cereals becomes pointless, because the absorbed P cannot be
retained, and P excretion simply shifts from fecal to urinary elimination. This explained the
observed 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. 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 1.0 to 0.5% 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. Due to the coinciding Ca and P demand for mineralization, Ca absorption was increased together with the increase of digestible P (Blaabjerg et al., 2010; Blaabjerg et al., 2015). Therefore, these studies suggested to use the ratio between Ca and digestible P (Ca:dP) instead of Ca:P to formulate the proper dietary Ca supply in pig diets.

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 ATTD of P of SBM is influenced by diet type, because SBM had a lower P digestibility when fed together with corn (24%) compared with barley (28%) or wheat (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). These results clearly demonstrate that additivity between feedstuffs can only be expected when no phytase is present. Interestingly, the ATTD of P of SBM calculated in the current study was quite high (45 to 58%) compared with the results of Fandrejewski et al. (1997) (24% to 37%) and Zhai and Adeola (2013) (36% to 38%). This difference may be due to e.g. the dissimilarity in the SBM grade or cultivar and soybean processing. The calculated ATTD 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. This also agreed with previous studies that observed a decrease of 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 ATTD of P of SBM in dry stored barley. Thus, barley seemed to be the bottleneck for improvements in ATTD of P in pigs, because the endogenous phytase in barley could degrade phytate and improve the calculated ATTD of P of SBM to the same extent as with addition of the enzyme combination. The numerical lower calculated ATTD of P of SBM (13% points) 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 ATTD of P of SBM when fed together with HMA stored barley to compensate for the decrease in endogenous enzymes activity after 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.

CONCLUSION

Overall, 35% moisture seems to be the optimum moisture level for HMA storage of 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. High moisture airtight storage of barley at 35% moisture (solely endogenous enzymes) degraded phytate releasing P for absorption and thereby enhanced the ATTD of P in the barley diet to the same extent as dry stored barley with enzyme combination (endogenous enzymes plus added enzymes) (49% vs. 45%, respectively). The inclusion of the enzyme combination to HMA stored barley resulted in a highest enhancement of ATTD of P in the barley diet (up to 70%). When barley was fed together with SBM, a similar but smaller enhancement of ATTD of P by HMA storage of barley with the enzyme combination was observed (up to 36%). The endogenous enzymes in dry stored barley degraded phytate and improved the calculated ATTD of P of SBM to the same extent as with addition of the enzyme combination. However, because of the decrease in endogenous enzyme activities during storage, the inclusion of the enzyme combination was necessary to enhance the calculated ATTD of P of SBM when fed together with HMA stored barley. Overall, the enhancement in ATTD 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 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.

LITERATURE CITED


Brown, E. M. 1991. Extracellular Ca\(^{2+}\) sensing, regulation of parathyroid cell function, and role of Ca\(^{2+}\) and other ions as extracellular (first) messengers. Physiol Rev. 71: 371-411


Humer, E., W. Wetscherek, C. Schwarz, and K. Schedle. 2014. Effects of maize conservation techniques on the apparent total tract nutrient and mineral digestibility and


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, PhytateP:Total P, nutrient solubility, phytase activity, and pH of barley after 49 d of storage in Exp. 1

<table>
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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.
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.
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

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>Enzyme</th>
<th>Dry storage</th>
<th>HMA storage</th>
<th>SEM</th>
<th>P-value S</th>
<th>S x E</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM, g/kg diet</td>
<td></td>
<td></td>
<td>861</td>
<td>666</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>861</td>
<td>667</td>
<td></td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total P, g/kg DM</td>
<td></td>
<td></td>
<td>3.38</td>
<td>3.31</td>
<td>0.06</td>
<td>0.778</td>
<td>0.266</td>
<td>0.410</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>3.25</td>
<td>3.29</td>
<td></td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>P solubility</td>
<td></td>
<td></td>
<td>0.23</td>
<td>0.77</td>
<td>0.01</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.35</td>
<td>0.87</td>
<td></td>
<td>p &lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytate P, g/kg DM</td>
<td></td>
<td></td>
<td>1.64</td>
<td>0.93</td>
<td>0.02</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>1.66</td>
<td>0.69</td>
<td></td>
<td>p &lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytate P:Total P</td>
<td></td>
<td></td>
<td>0.49</td>
<td>0.28</td>
<td>0.01</td>
<td>0.006</td>
<td></td>
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<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.51</td>
<td>0.21</td>
<td></td>
<td>p &lt; 0.103</td>
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<tr>
<td>Phytase activity, FTU/kg DM</td>
<td></td>
<td></td>
<td>420</td>
<td>320</td>
<td>140</td>
<td>p &lt; 0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>1,600</td>
<td>980</td>
<td></td>
<td>p &lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylanase, U/kg DM</td>
<td></td>
<td></td>
<td>&lt;100</td>
<td>&lt;100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>1,630</td>
<td>2,550</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-glucanase activity, BU/kg DM</td>
<td></td>
<td></td>
<td>4,290</td>
<td>3,120</td>
<td>3,430</td>
<td>0.105</td>
<td>0.002</td>
<td>0.175</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>24,500</td>
<td>13,140</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td>5.78</td>
<td>6.02</td>
<td>0.02</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>5.80</td>
<td>6.14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary treatment</th>
<th>Barley diet</th>
<th>Barley-SBM diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Item</td>
<td></td>
<td>Dry storage</td>
<td>HMA storage</td>
</tr>
<tr>
<td>Item</td>
<td></td>
<td>- E + E</td>
<td>- E + E</td>
</tr>
<tr>
<td>Ingredient, % as fed-basis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean meal</td>
<td>30.16</td>
<td>30.16</td>
<td>25.65</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.35</td>
<td>0.35</td>
<td>0.27</td>
</tr>
<tr>
<td>DM, %</td>
<td>87</td>
<td>87</td>
<td>67</td>
</tr>
<tr>
<td>CP, g/kg DM</td>
<td>114</td>
<td>116</td>
<td>118</td>
</tr>
<tr>
<td>Total Ca, g/kg DM</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Total P, g/kg DM</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>Phytate P, g/kg DM</td>
<td>1.8</td>
<td>1.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Na, g/kg DM</td>
<td>1.8</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Cl, g/kg DM</td>
<td>3.5</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Phytase activity, FTU/kg DM</td>
<td>400</td>
<td>1,870</td>
<td>280</td>
</tr>
</tbody>
</table>

1 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).

3 CP was calculated as N (g/kg DM) x 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, apparent total tract digestibility (ATTD) of DM and P, and the balance of P and Ca in barley diet in Exp. 2 (LS means ± SEM)

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary treatment</th>
<th></th>
<th></th>
<th>SEM</th>
<th>P-value²</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry stored barley</td>
<td>HMA stored barley</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-E</td>
<td>+E</td>
<td>-E</td>
<td>+E</td>
<td>S</td>
<td>E</td>
<td>S x E</td>
</tr>
<tr>
<td>DM intake, g/d</td>
<td>1.162</td>
<td>1.167</td>
<td>1.172</td>
<td>1.142</td>
<td>0.46</td>
<td>0.658</td>
<td>0.092</td>
</tr>
<tr>
<td>ATTD of DM, %</td>
<td>87.4</td>
<td>89.0</td>
<td>87.9</td>
<td>88.0</td>
<td>0.46</td>
<td>0.658</td>
<td>0.092</td>
</tr>
<tr>
<td>P balance, g/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intake</td>
<td>3.79</td>
<td>3.91</td>
<td>3.91</td>
<td>3.77</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feces</td>
<td>2.28</td>
<td>1.66</td>
<td>1.58</td>
<td>1.21</td>
<td>0.12</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Urine</td>
<td>1.27</td>
<td>1.83</td>
<td>1.71</td>
<td>2.19</td>
<td>0.13</td>
<td>0.005</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Net absorption</td>
<td>1.51</td>
<td>2.24</td>
<td>2.33</td>
<td>2.56</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retention</td>
<td>0.25</td>
<td>0.41</td>
<td>0.62</td>
<td>0.37</td>
<td>0.15</td>
<td>0.249</td>
<td>0.761</td>
</tr>
<tr>
<td>ATTD of P, %</td>
<td>40.0</td>
<td>57.8</td>
<td>59.6</td>
<td>67.9</td>
<td>2.32</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Digestible P, g/kg DM</td>
<td>1.31</td>
<td>1.93</td>
<td>1.99</td>
<td>2.24</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca balance, g/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intake</td>
<td>0.57</td>
<td>0.61</td>
<td>0.62</td>
<td>0.59</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feces</td>
<td>1.05</td>
<td>0.63</td>
<td>0.65</td>
<td>0.43</td>
<td>0.06</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Urine</td>
<td>0.04</td>
<td>0.04</td>
<td>0.05</td>
<td>0.02</td>
<td>0.02</td>
<td>0.780</td>
<td>0.381</td>
</tr>
<tr>
<td>Net absorption</td>
<td>-0.48</td>
<td>-0.02</td>
<td>-0.03</td>
<td>0.16</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retention</td>
<td>-0.53</td>
<td>-0.07</td>
<td>-0.08</td>
<td>0.16</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATTD of Ca, %</td>
<td>-84.9</td>
<td>-2.91</td>
<td>-5.08</td>
<td>26.7</td>
<td>8.06</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.
**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, apparent total tract digestibility (ATTD) of DM, P and Ca, and the balance of P and Ca in barley-soybean meal (SBM) diet in Exp. 2 (LS means ± SEM)

| Item                        | Dietary treatment |    |    |    | SEM |  |  |  |
|-----------------------------|-------------------|----|----|----|-----||--|--|--|
|                             | Dry stored barley-SBM | HMA stored barley-SBM | +E | -E | -E | +E |
| DM intake, g/d              |                   |    |    |    |     |  |  |  |
| 1.223                       | 1.233              | 1.177| 1.189| 1.6 | 0.015| 0.515| 0.928|
| ATTD of DM, %               |                   |    |    |    |     |  |  |  |
| 86.9                        | 87.2               | 87.5| 88.1| 0.50| 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| < 0.001| < 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|
| ATTD of P, %                |                   |    |    |    |     |  |  |  |
| 45.8                        | 57.0               | 52.3| 62.3| 1.83| < 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|
| ATTD of Ca, %               |                   |    |    |    |     |  |  |  |
| 1.06                        | 24.4               | 13.7| 40.3| 5.88| < 0.001| < 0.001| 0.556|

- 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.
\[ P-value: S = \text{storage effect (dry storage vs. HMA storage at 35% moisture)}, \ E = \text{enzyme combination effect (without vs. with enzyme combination)}; \ S \times E = \text{the interaction between storage and enzyme combination}. \]
Table 6. Apparent total tract digestibility (ATTD) 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

<table>
<thead>
<tr>
<th>Basal diet¹</th>
<th>ATTD of P in barley diet (%)</th>
<th>ATTD of P in barley-SBM diet (%)</th>
<th>Calculated ATTD of P in SBM (%)</th>
<th>SEM</th>
<th>Mean</th>
<th>P-value²</th>
<th>S x E</th>
<th>S</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry stored barley</td>
<td>40.0</td>
<td>45.8</td>
<td>57.8</td>
<td>4.19</td>
<td>58.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.080</td>
<td>0.043</td>
<td>0.057</td>
<td></td>
</tr>
<tr>
<td>Dry stored barley + E</td>
<td>57.8</td>
<td>57.1</td>
<td>58.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMA barley</td>
<td>59.6</td>
<td>52.3</td>
<td>44.6</td>
<td></td>
<td>51.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMA barley + E</td>
<td>76.9</td>
<td>62.3</td>
<td>57.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Basal 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).

²P-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.
Manuscript III

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

M. A. Ton Nu, K. Blaabjerg, and H.D. Poulsen

Submitted to Animal for publication
Effects of high moisture airtight stored barley with exogenous enzymes on protein and amino acid digestibility in barley fed to pigs alone or together with soybean meal

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Short title: N and AA digestibility of high moisture stored barley

Abstract

The objective was to evaluate the effect of storage of rolled barley (dry storage at 13% moisture vs. high moisture airtight (HMA) storage at 35% moisture for 49 days) without or with an enzyme combination of phytase, xylanase, β-glucanase, and protease on apparent total tract digestibility (ATTD) and apparent ileal digestibility (AID) of nitrogen (N) and AID of amino acids when the barley was fed to pigs alone (barley diet) or in combination with 30% soybean meal (barley-SBM diet). Forty-eight female pigs (43 ± 2 kg) were housed in metabolism crates and fed one of the eight diets for 15 days: 5 days for adaptation, 7 days for total, but separate collection of faeces and urine, and 3 days of feeding the diets with 0.3% of chromium oxide as a marker. On day 15, pigs were slaughtered three hours after the morning feeding, and ileal digesta was collected. HMA storage of barley increased the solubility of N (13% points) and protein (4% points) compared with dry storage. Adding the enzyme combination to HMA stored barley enhanced N and P solubility to a greater extent (4% and 2% points, P<0.05) compared with no enzyme addition, whereas the enzyme combination had no effect on dry stored barley. Although storage had no effect on ATTD and AID of N (P>0.05), HMA storage of barley increased AID of Met and Arg (5% points, P<0.05). The enzyme combination enhanced AID of N (7% points), total amino acids (6% points), and Lys (7% points) (P<0.05). The inclusion of the enzyme combination during HMA storage of barley resulted in the highest increase in AID of Lys, Met, Arg, (10-15%), and in the digestible amounts of Lys, Met+Cys, Ile, and Phe (7-17%) of the barley diets compared with the dry stored barley (P<0.05). However, ATTD and AID of N as well as AID of amino acids were not influenced by barley stored dry or...
HMA (without or with enzyme combination) when fed together with SBM. Feeding HMA stored barley together with SBM increased the amounts of digestible Lys and Arg ($P<0.05$).

In conclusion, HMA storage of rolled barley with enzyme combination is a possibility to increase the protein value of cereals and thereby reduce the inclusion rate of other protein feedstuffs resulting in a decrease in N excretion.

**Keywords:** cereal, storage, amino acid, enzyme, pigs

**Implications**

High moisture airtight (HMA) storage and an enzyme combination of phytase, xylanase, β-glucanase, and protease enhanced the protein digestibility and the digestible amount of some indispensable amino acids (AA) – especially the non-commercially available AA (Ile, Leu, Phe and Arg) – due to increased solubility of N and protein. This highlights a potential for improving the nutritional value of barley and other cereals not only on P but also on protein and thereby reduce the supplementation rate of protein feedstuffs and the N excretion. Protein solubility seems to be a promising indicator to predict the AA and protein digestibility of barley.

**Introduction**

Intensive pig production is of environmental concern due to the high excretion of nitrogen (N) in manure which can leach to the ground and surface water and cause ammonia emission (Aarnink and Verstegen, 2007). This is mainly due to the low N digestibility and the amino acid profile of the cereals which often constitute above 70% of the diet. The apparent ileal digestibility (AID) of N in barley averaged 69% (Spindler et al., 2014), whereas the AID of protein feedstuffs like soybean meal (SBM) approximates 86% (Brestensky et al., 2013). The high amount of dispensable amino acids in barley also leads to a high content of amino acids that are deaminated and excreted as urea in urine. Thus, the cereal part seems to be one of the drawbacks for further improvement of N digestibility and utilization in pig diets.

Non-starch polysaccharides (NSP) and phytate encapsulate or bind protein in the grain matrix of barley. Thus, in order to increase the N digestibility of barley, it is a challenge to degrade the complex structure of these components. This requires a combination of various enzymes that are not secreted or secreted in negligible amounts by the pig’s digestive tract. Cereals like barley, however, possess endogenous enzymes like xylanases (Simpson et al., 2003), β-glucanases (Ribeiro et al., 2011), phytases (Viveros et al., 2000), and proteases (Jones, 2005) which are also available as commercial enzymes. However,
enzymes require moist conditions to be activated and sufficient time to fully degrade their substrates. Furthermore, endogenous enzymes in cereals and added microbial enzymes encounter many challenges like low pH in the stomach and proteolytic activities of other enzymes along the gastrointestinal tract.

High moisture airtight (HMA) storage of cereals creates moist conditions that may activate the endogenous enzymes and degrade the grain matrix before feeding. As such, HMA storage increased the content of soluble N in maize (Baron et al., 1986) and barley (Aman et al., 1990). Furthermore, HMA storage of barley at 35% moisture enhanced both N and protein solubility to a greater extent compared with moisture levels below 30% as well as the inclusion of an enzyme combination (phytase, xylanase, β-glucanase, and protease) augmented the increasing effect of HMA storage (M.A Ton Nu, unpublished data). HMA storage also enhanced the apparent total tract digestibility (ATTD) of N of wheat at 25% moisture (Pieper et al., 2011), and the AID of the indispensable amino acids of barley at 29% moisture (Weltzien and Aherne, 1987). However, it remains unknown if HMA storage of barley at 35% moisture without or with enzyme combination affects the digestibility of N, and if the AID of the indispensable and dispensable amino acids is affected to the same degree.

It is hypothesized that HMA storage of barley at 35% moisture increases the ATTD and AID of N and the AID of amino acids in pigs compared with dry storage, and that a further increase is achieved by storing the barley together with the added enzyme combination promoting the degradation of the grain matrix. It is also hypothesized that the activated endogenous enzymes in HMA stored barley and the added enzyme combination increase the ATTD and AID of N and the AID of amino acids of SBM when fed together with HMA stored barley. The aim of this study is to investigate the effect of storage (HMA stored vs dry stored) of rolled barley without or with addition of the enzyme combination (phytase, xylanase, β-glucanase and protease) on the ATTD and AID of N and the AID of amino acids in pigs fed barley alone or in combination with SBM but without crystalline amino acids.

Material and methods

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

Feedstuff preparation and diet formulation
Barley (winter cultivar, *Zephyr*) was ground by a roller mill (Skiold crushers KB 200, SKIOLD A/S, Sæby, Denmark) and stored either at 13% moisture in plastic bags (dry storage) or at 35% moisture in airtight bags (HMA storage) for 49 days. Before storage, rolled barley was supplemented without or with an enzyme combination of phytase (Natuphos 5000G, BASF SE, Ludwigshafen, Germany) at 1000 FTU/kg feed, xylanase (Danisco Xylanase 8000 G, Danisco Animal Nutrition, Marlborough, England) at 4000 U/kg feed, β-glucanase (ECONASE Barley P700, AB Vista, Marlborough Wiltshire, United Kingdom) at 17500 BU/kg feed, and protease (RONOZYME ProAct CT, DSM Nutritional Products Ltd, Basel, Switzerland) at 15000 PROT/kg feed. The HMA stored barley was prepared as follows: addition of water (with or without enzyme combination) to rolled barley to adjust the moisture content to 35%, sealing of the bags (400 kg/bag), and mounting of a valve allowing the refilling of CO₂ regularly to ensure airtight conditions during storage. Before storage (day 0) and after 49 days of storage, samples were collected in triplicate and immediately frozen to stop further enzymatic activities. Thereafter, the dry stored barley and the HMA stored barley without or with the enzyme combination were ground separately by a hammer mill (3.5-mm screen size).

The experimental diets (Table 1) were formulated to fulfill the Danish recommendations for growing-finishing pigs (45 to 105 kg) (Tybirk *et al.*, 2014) except for amino acids, phosphorus (P), and calcium (Ca). No crystalline amino acids, mineral phosphate, or Ca were added. The diets consisted of either barley (dry stored or HMA stored barley with or without enzyme combination) as the sole ingredient or a combination of barley and SBM (Table 1). Sodium chloride (NaCl) was added to all diets to fulfill the sodium requirement of pigs. For the HMA stored barley-SBM diets, the SBM was mixed with HMA stored barley immediately before feeding. Diets fed to the pigs during the final three days of the experimental period were supplemented with 3 g Cr₂O₃/kg diet as-fed basis in order to measure the AID of N and amino acids.

**Animals**

Forty-eight female pigs (43 ± 2 kg) were randomly assigned to the barley diets or to the barley-SBM diets in a 2 x 2 factorial arrangement of storage (dry storage vs. HMA storage at 35% moisture) and enzyme combination (without vs. with). The pigs were individually housed in metabolic cages (70 x 135 cm) and were fed twice daily (0800 h and 1400 h). Feed consumption and feed refusals were recorded daily. The experimental period consisted of 15 days: 5 days for adaptation, 7 days for total collection of urine and feces,..
and 3 days of feeding diets supplemented with chromium (III) oxide ($\text{Cr}_2\text{O}_3$). On day 5, the pigs were fitted with urine bladder catheters to collect urine and feces separately. Urine samples were collected in the 5-l containers with 40 ml of a 30% $\text{H}_2\text{SO}_4$ addition to avoid N loss. On day 15, pigs were slaughtered three hours after the morning feeding. Ileal digesta was collected 3 m proximal to the ileocecal junction. All samples were frozen immediately after collection.

**Chemical analysis**

Dry matter content (DM) of barley (day 0 and day 49), diets, feces, and ileal digesta was determined by oven drying at 103°C for 20 hours. Barley was analysed for total N, soluble N, and soluble protein. Soluble N comprised total N (protein and non-protein N) solubilized in water. Soluble protein included polypeptides (tripeptides or larger) and protein solubilized in water. Soluble N and soluble protein were measured in extracted supernatants after mixing the barley samples with water at a ratio of 1:2.3 for 10 minutes as described by M.A. Ton Nu (unpublished results, Paper 1). Soluble protein was measured by the biuret method using a Roche cobass c 111 analyser (Roche Diagnostics GmbH, Mannheim, Switzerland). Total N and soluble N of barley were measured by a Kjeltec AUTO 2400 Analyzer System (FOSS, Hillerød, Denmark) according to the Kjeldahl method 978.02 (AOAC, 2000). Total N was analysed in diets, feces, urine, and ileal digesta by the Dumas method (Hansen, 1989). The measurement of $\text{Cr}_2\text{O}_3$ in diets, feces, and ileal digesta was performed on wet materials by the method of Schürch et al. (1950). Amino acids in diets and ileal digesta were separated by ion exchange chromatography and quantified by photometric detection after ninhydrin reaction (European Commission, 1998).

**Calculations**

The solubility of N and protein of barley and CP of barley and diets were calculated as follows:

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

Where: $k$ is the nitrogen-to-protein conversion factor. Different feedstuffs vary in the N:protein ratio, so a specific $k$ factor of 5.45 was used to calculate CP of barley (Mariotti et al., 2008), whereas the $k$ factor of 6.25 was used to calculate CP of the barley-SBM diets.

$$\text{N solubility} = \frac{\text{Soluble N (g/kg DM)}}{\text{Total N (g/kg DM)}}$$

$$\text{Protein solubility} = \frac{\text{Soluble protein (g/kg DM)}}{\text{CP (g/kg DM)}}$$

**Statistical analysis**
SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA) was used for the statistical analyses. All data are presented as least square means (LS means) and standard error of means (SEM). P-values are considered significant if $P<0.05$ and the tendencies are declared at $0.05 \leq P \leq 0.1$. Results of CP and the solubility of N and protein of barley were analysed using the GLM procedure. The statistical model included the main effect of storage (dry storage vs. HMA storage at 35% moisture), the enzyme combination (without vs. with), and their interaction. Results of ATTD and AID of N, AID of amino acids, and N balance were analysed for the barley and the barley-SBM diets separately using the MIXED procedure. The model included the main effect of storage (dry storage vs. HMA storage at 35%), enzyme combination (without vs. with), and their interaction. Each pig served as the experimental unit for all responses. Treatment differences were separated by the PDIFF option of SAS software.

**Results**

**Crude protein and the solubility of N and protein in stored barley**

The CP content of HMA stored barley was slightly higher (1% unit) compared to that of dry stored barley at day 49 ($P=0.043$, Table 2). The solubility of N and protein in barley was affected by the interaction between storage and enzyme combination ($P=0.017$ and $P=0.022$, respectively). This is because HMA storage of barley with the enzyme combination enhanced to a greater extent the solubility of N (17% points) and protein (6% points) compared with no addition of enzyme combination (13% and 4% points, respectively) ($P<0.05$). In contrast, the enzyme combination had no effect on the N and protein solubility of dry stored barley ($P>0.05$).

**N balance and the digestibility of N and amino acids in barley diets**

The content of DM, CP and amino acids was similar among treatments in barley diets (Table 3). No interaction between storage and enzyme combination on the ATTD and the AID of N, the AID of amino acids, and the digested amounts of amino acids was observed when pure barley was fed the pigs (Tables 4, 5, and 6). Furthermore, HMA storage did not enhance the ATTD and AID of N in barley ($P>0.05$, Table 4), whereas HMA storage enhanced the AID of some of the indispensable amino acids like Met (5% points, $P=0.031$) and Arg (5% points, $P=0.044$) and tended to enhance the AID of Phe (4% points, $P=0.076$) compared with dry storage of barley (Table 5). Also, HMA storage increased the amounts of digestible Ile (8%, $P=0.041$) and Phe (6%, $P=0.041$) (Table 6). In contrast, HMA storage did not affect ($P>0.05$) the AID of the dispensable amino acids (Tables 5 and 6).
Irrespective of storage method, the enzyme combination enhanced the AID of N in barley by 7% points ($P=0.028$) and tended to increase the urinary N excretion by 11% ($P=0.095$) compared with no enzyme addition (Table 4). Accordingly, AID of total amino acids increased by 5% points when the enzyme combination was added compared with no enzyme addition ($P=0.043$, Table 5). The explanation is that the enzyme combination enhanced the average AID of dispensable amino acids (5% points, $P=0.046$) and tended to increase the average AID of indispensable amino acids (4% points, $P=0.085$) (Table 5). Consequently, the enzyme combination increased the AID of Lys (7% points, $P=0.045$) and Ala (9% points, $P=0.019$) and tended to enhance the AID of Ile (5% points, $P=0.068$), Leu (5% points, $P=0.074$), Phe (4% points, $P=0.075$), and most of the dispensable amino acids by 4-7% points ($0.5 \leq P \leq 0.1$) except Gly and Pro (Table 5). The enzyme combination enhanced the total amount of digestible dispensable amino acids by 10% ($P=0.030$) and tended to increase the total amount of digestible indispensable amino acid by 7% ($P=0.060$) (Table 6). With the enzyme combination, the digestible content of amino acids was increased in Lys (10%), Met+Cys (8%), Phe (6%), Ala (16%), Asp (13%), Cys (10%), and Glu (6%) ($P<0.05$) and tended to increase in Met, His, Val, and Ser (5-9%) ($0.5 \leq P \leq 0.1$) (Table 6).

N balance and the digestibility of N and amino acids in barley-SBM diets

The content of indispensable amino acids was slightly higher in HMA stored barley compared with dry stored barley in barley-SBM diet (Table 3). The interaction between storage and enzyme combination influenced N absorption ($P=0.026$) and digestible N ($P=0.002$) in the barley-SBM diets (Table 7). The explanation is that dry-stored barley-SBM diets resulted in a higher N absorption of 2.42 g/day compared with HMA stored barley-SBM diets ($P=0.008$) when no enzyme combination was added, whereas this difference was not observed in diets with the enzyme combination ($P=0.659$). Additionally, a higher content of digestible N (5%) was observed in HMA stored barley-SBM diets compared with dry stored barley-SBM diets when adding the enzyme combination ($P=0.010$), whereas no difference appeared in barley-SBM diets without enzyme addition ($P=0.413$) (Table 7).

High moisture airtight storage of barley and the enzyme combination had no effect on the AID of amino acids in barley-SBM diets ($P>0.05$, Table 8). High moisture airtight-stored barley, however, increased the amounts of digestible Lys (7%, $P=0.007$) and Arg (4%, $P=0.027$) compared with dry stored barley (Table 9). The DM intake was about 4% lower in pigs fed the dry stored compared to the HMA stored barley. However, the inclusion of DM intake as a covariate in the statistical model revealed no change in all parameters
compared with the model without the covariate except in N retention. The correction for DM intake displayed that the enzyme combination decreased the N retention by 8% compared with no addition of enzymes ($P=0.042$).

**Discussion**

The current N solubility of barley after 49 days of dry storage was in the range (0.17-0.21) reported by Aman *et al.* (1990) in barley harvested at 36%, 25%, and 23% moisture. High moisture airtight storage of barley at 36% moisture with *lactobacilli* and yeast for one year increased the N solubility to 0.30 (Aman *et al.*, 1990). This is similar to the current increase of N solubility of HMA stored barley at 35% moisture without the enzyme combination at day 49. High moisture airtight storage of barley with the enzyme combination increased the N solubility to 0.34. This implies that the enzyme combination degraded the grain matrix of barley and enhanced the N solubility to a greater extent compared with solely endogenous enzymes of barley.

As expected, the enzyme combination did not affect the N and protein solubility in dry stored barley. The present protein solubility in barley before storage and in dry stored barley at day 49 was slightly higher compared with the observed values (0.12-0.14) of two winter barley cultivars (Finlissa and Zephyr) in the study by Christensen *et al.* (2014). High moisture airtight stored barley with the enzyme combination in the present study led to a similar increase of protein solubility (0.22) as observed in barley soaked for 48 hours at 20°C (Christensen *et al.*, 2014). The moist condition during HMA storage and soaking of the cereals activates enzymes that decompose the grain matrix and mobilize protein. However, the higher water content and the higher temperature during soaking obviously enhanced the solubilisation of protein at a higher rate (Christensen *et al.*, 2014) compared with HMA storage.

In the current study, HMA storage of barley without and with enzyme combination enhanced the N solubility (13% and 17% points, respectively) to a greater extent compared with the protein solubility (4% and 6% points, respectively). This resulted in a lower proportion of soluble protein relative to soluble N in HMA stored barley compared with dry stored barley, possibly because the solubilized protein and polypeptides (tripeptides or larger) were further degraded to dipeptides and amino acids (not included in the soluble protein fraction in the current analysis technique) leading to the observed decrease in soluble protein and the increase in soluble N.
A limited number of studies have investigated the effect of HMA storage on the ATTD of N and the results are inconsistent. Humer et al. (2013) found a lower ATTD of N in pigs fed HMA stored maize (24% moisture) compared with dry stored maize. The lack of effect of HMA storage on the ATTD of N in barley in the current study is similar to previous studies of barley and triticale stored at 25-35% moisture and wheat at 30% and 35% moisture (Hackl et al., 2010; Pieper et al., 2011) and maize at 30% moisture (Humer et al., 2014). Poulsen et al. (2012) reported a tendency to enhance the ATTD of N by 3% points in pigs fed a HMA-stored barley-wheat diet (18% moisture). Also, HMA storage of wheat at 25% and 35% moisture enhanced the ATTD of N by 2% and 1% points in pigs, respectively (Pieper et al., 2011). Though no effect on the AID of N, HMA stored barley increased the AID of Met, Arg, and Phe in the current study and the AID of Lys, Ile, and Val in the study by Weltzien and Aherne (1987). Similar improvements in standardized ileal digestibility (SID) of Lys, Met, Thr, Leu, Phe, and Arg were reported in HMA stored wheat at 30% moisture, whereas HMA storage of barley at 30% moisture decreased the SID of Lys and His (Hackl et al., 2010).

These inconsistent effects of HMA storage on the ATTD and AID of N and the AID of amino acids in cereals might be due to the differences in e.g. moisture levels, storage time, cereal types, and cultivars. Moreover, barley contains a higher ratio of β-glucans – the highly soluble NSP fraction that is easily degraded by microbial enzymes in the distal segment of the small intestine compared to wheat (Bach Knudsen and Hansen, 1991). Thus, the degradation of NSP in barley by the microorganisms in the small intestine may enhance the release of protein from the grain matrix and thereby conceal the positive effect of HMA storage of barley and enzyme combination on pigs.

The increase in protein solubility of barley before feeding is expected to result in a higher amount of digestible protein and amino acids in diets. Soaking the same barley cultivar (Zephyr) as used in the current study increased the protein solubility by 15-22% points and the ATTD of N by 3% points (Christensen, 2013). Thus, the current, limited increase in the ATTD and AID of N and the AID of amino acids in HMA stored barley may be partly explained by the small increase of protein solubility (4-6% points) in HMA stored barley compared with dry stored barley. This indicates the potential to use protein solubility as a rapid indicator to predict the N and amino acid digestibility in pigs. Moreover, the enzyme combination enhanced the AID of N and amino acids in dry stored barley to the same extent as in HMA stored barley. Thus, the increased protein and N solubility caused by HMA storage and enzyme combination before feeding seems to have a corresponding small
impact on the AID of N and amino acids. As such, the enzyme combination seems to
solubilize protein and amino acids of dry stored barley in the proximal part of the digestive
tract to the same extent as that of HMA stored barley.

At present, Lys, Thr, Met, Trp, and Val are the commercially available crystalline amino
acids. After fulfilling the requirements of Lys and Thr by crystalline amino acids, Ile, Met, His,
Leu, Val, and Phe become the next limiting amino acids of barley. Thus, the digestible
amounts of the non-commercially available indispensable amino acids (Ile, His, Leu, Phe, Arg) are the limiting amino acids to improve N utilization because these amino acids can
only be provided by feedstuffs.

Non-starch polysaccharides encapsulate 26–28% of total N, 40% of Lys, 36% of Thr, and
33% of Met+Cys in barley (Rybka et al., 1992). Thus, the present enhancement in the AID of
indispensable amino acids in barley by HMA storage and the enzyme combination
indicates that the endogenous enzymes in barley and the added microbial enzymes may
hydrolyse NSP and phytate to release the encapsulated amino acids. The addition of a
combination of xylanase, β-glucanase, and protease to a barley diet also enhanced the
AID of N (7% points), total NSP (14% points), and all indispensable amino acids (9% points;
Yin et al. (2001). Yin et al. (2001) stated that the inclusion of protease in the enzyme
combination had no extra effect on the nutrient digestibility of protein feedstuffs compared
with the addition of xylanase or β-glucanase. A literature review by Adeola and Cowieson
(2011) reported that the effect of phytase on AID of amino acids is contradictory, but the
increase in AID of amino acids by microbial phytase may be 6–7% in pigs. The AID of Cys,
Thr, Ser, Pro, and Gly increased after microbial phytase addition, whereas the AID of Met,
Arg, Glu, and Lys responded less (Adeola and Cowieson, 2011).

The tendency to a higher urinary N excretion in pigs fed barley diets with the enzyme
combination may be due to the increased absorption of the dispensable and to some
extent also the indispensable amino acids which are deaminated and excreted via urea.
The increase in urinary N excretion may be related to the increased NSP degradation in
barley caused by the enzyme combination. The decrease in fermentable carbohydrates
like β-glucan also reduces the secretion of urea from the blood due to lowered microbial
growth resulting in lower microbial protein synthesis and higher reabsorption of ammonia
from the colon shifting the N excretion from feces to urine (Canh et al., 1997, Zervas and
Zijlstra, 2002). The current results also agree with the higher ratio of urinary N to feces N
observed in pigs fed HMA stored maize (Humer et al., 2014). Urea is the main source of
ammonia emission from pig manure, whereas only a small part originates from the
degradation of protein in feces (Aarnink et al., 1993; Aarnink and Verstegen, 2007).
Therefore, the shift of N excretion from feces to urine, all things being equal, contributes to
a higher ammonia emission. However, the enhancement in the AID of indispensable
amino acids may lead to a reduction in ammonia emission by a proper use of crystalline
AAs and complementary CP.

High moisture airtight storage of barley also enhanced the content of digestible Lys and
Arg – partly because of a higher Lys and Arg content in HMA stored barley-SBM diets
compared with dry stored barley-SBM diets. The lack of effect of HMA storage on the AID of
N and amino acids of the barley-SBM diets is most likely because the activity of enzymes
diminished during HMA storage (M.A Ton Nu, unpublished data – Paper 2). Moreover, the
short time for digestion in the digestive tract may restrict the effect of the enzyme
combination on enhancing AID of N and amino acids of SBM.

Conclusion

High moisture airtight storage of rolled barley at 35% moisture for 49 days enhanced the
solubility of N (73%) and protein (25%) compared with dry storage. The inclusion of the
enzyme combination (phytase, xylanase, β-glucanase, and protease) during HMA storage
of barley increased the solubility of N and protein to a greater extent (13% and 10% points,
respectively) than without the enzyme addition. However, the enzyme combination did not
increase N and protein solubility of dry stored barley. Storage (HMA storage vs. dry storage)
per se had no effect on ATTD and AID of N in barley whereas the inclusion of the enzyme
combination enhanced AID of N (12% points) in barley diets compared with no enzyme
addition. Furthermore, storage of barley (HMA storage vs. dry storage) without and with the
enzyme combination did not affect the overall ATTD and AID of N in pigs when fed
together with SBM. Interestingly, the enzyme combination significantly enhanced the
average AID of dispensable amino acids (8%) and tended to increase the average AID of
indispensable amino acids (6%) in barley diets compared with no enzyme addition. Thus
the addition of the enzyme combination to HMA stored rolled barley resulted in the highest
improvement in AID of indispensable amino acids (Lys, Met, and Arg, 10-15%) and in the
amounts of digestible indispensable amino acids (Lys, Met+Cys, Ile, and Phe, 7-17%) in
barley diets compared with dry stored barley. The feeding of HMA stored barley together
with SBM increased the amount of digestible Lys (7%) and Arg (4%) in barley-SBM diets
compared with dry stored barley. The enhancement in AID of indispensable amino acids
of barley by HMA storage with enzyme combination revealed the possibility of improving
the nutritional quality of the cereal protein and thereby to reduce the need for protein
supplementation and N excretion. Furthermore, protein solubility seems to be a rapid and
reliable indicator to predict AID of N and amino acids of cereals in pigs. Moreover, storage
conditions (e.g. pH and temperature) and factors like cereal type and cultivar need to be
studied further to optimize the use of HMA storage and enzyme combination as an
approach to increase the nutritional quality of cereal protein and to reduce N excretion.

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References

Aarnink AJA and Verstegen MWA 2007. Nutrition, key factor to reduce environmental load from pig
production. Livestock Science 109, 194-203.

Aarnink AJA, Hoeksma P and van Ouwerkerk ENJ 1993. Factors affecting ammonium
concentration in slurry from fattening pigs. In Proceedings of the 1st International Symposium on
nitrogen flow in pig production and environmental consequences, Wageningen, The Netherlands,
pp. 413-420.

Adeola O and Cowieson AJ 2011. BOARD-INVITED REVIEW: Opportunities and challenges in using
exogenous enzymes to improve nonruminant animal production. Journal of Animal Science 89,
3189-3218.

Aman P, Pettersson D and Graham H 1990. Chemical and nutritional-evaluation of airtight storage
of high-moisture barley and high-moisture barley treated with lactobacilli or lactobacilli and yeast.

Animal Feed Science and Technology 29, 223-235.

analysis, pp. 18-19, Association of Official Analytical Chemists, Arlington, VA, USA.

Bach Knudsen KE and Hansen I 1991. Gastrointestinal implications in pigs of wheat and oat

Baron VS, Stevenson KR and Buchanansmith JG 1986. Proteolysis and fermentation of grain-corn
ensiled at several moisture levels and under several simulated storage methods. Canadian Journal
of Animal Science 66, 451-461.

Brestensky M, Nitrayova S, Patras P and Heger J 2013. Standardized ileal digestibilities of amino
acids and nitrogen in rye, barley, soybean meal, malt sprouts, sorghum, wheat germ and broken
rice fed to growing pigs. Animal Feed Science and Technology 186, 120-124.

Christensen JB 2013. Exploiting the enzyme potential of cereals in liquid feed for pigs: possibilities and limitations. Thesis PhD, Aarhus University, Aarhus, Denmark.


Table 1. Ingredients of the experimental diets (% as feed basis)

<table>
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<tr>
<th>Dietary treatment¹</th>
<th>Dietary treatment²</th>
<th>Diet</th>
<th>Barley diet</th>
<th>Barley-SBM diet</th>
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<td>Storage</td>
<td>Dry storage</td>
<td>HMA storage</td>
<td>Dry storage</td>
<td>HMA storage</td>
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<tr>
<td>Enzymes</td>
<td>- E</td>
<td>+ E</td>
<td>- E</td>
<td>+ E</td>
</tr>
<tr>
<td>Dry stored barley</td>
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<td>99.65</td>
<td>69.49</td>
<td>69.49</td>
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<td>HMA stored barley</td>
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<td>99.73</td>
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<tr>
<td>Soybean meal</td>
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<td>30.16</td>
<td>25.65</td>
<td>25.72</td>
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<td>Sodium chloride</td>
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<tr>
<td></td>
<td>0.27</td>
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</table>

¹Rolled barley was dry stored (dry storage) or high moisture airtight (HMA) stored at 35% moisture (HMA storage) without (-E) or with (+E) the combination of phytase, xylanase, β-glucanase, and protease for 49 days, ground and then mixed without (barley diet) or with soybean meal (barley-SBM diet).
Table 2. Effects of storage (dry vs. high moisture airtight (HMA) storage at 35% moisture) and enzyme combination on CP, nitrogen (N), and protein solubility of barley after 49 days of storage

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary treatment</th>
<th>SEM</th>
<th>P-value</th>
<th>S</th>
<th>E</th>
<th>S x E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme</td>
<td>Dry stored barley</td>
<td>HMA stored barley</td>
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</tr>
<tr>
<td>CP, g/kg DM</td>
<td>- E</td>
<td>96</td>
<td>98</td>
<td>0.53</td>
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<td>0.740</td>
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<tr>
<td></td>
<td>+E</td>
<td>97</td>
<td>97</td>
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<tr>
<td>N solubility</td>
<td>-E</td>
<td>0.17</td>
<td>0.30</td>
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<td></td>
<td>+E</td>
<td>0.17</td>
<td>0.34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein solubility</td>
<td>-E</td>
<td>0.16</td>
<td>0.20</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>+E</td>
<td>0.16</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Rolled barley was dry stored or high moisture airtight (HMA) stored at 35% moisture without (-E) or with (+E) the combination of phytase, xylanase, β-glucanase, and protease for 49 days.

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

3CP was calculated as N (g/kg DM) x 5.45, where 5.45 is the specific nitrogen-to-protein conversion factor for barley (Mariotti et al., 2008).
### Table 3. Dry matter (DM, g/kg), CP, and amino acids (AA) contents of experimental diets (g/kg DM)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Dietary treatment¹</th>
<th>Barley diet</th>
<th>Barley-SBM diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry stored barley</td>
<td>HMA stored</td>
<td>Dry stored barley</td>
</tr>
<tr>
<td>DM</td>
<td>- E</td>
<td>+ E</td>
<td>- E</td>
</tr>
<tr>
<td>CP²</td>
<td>99</td>
<td>101</td>
<td>103</td>
</tr>
</tbody>
</table>

Indispensable AA, g/kg DM

<table>
<thead>
<tr>
<th></th>
<th>Lys</th>
<th>Met</th>
<th>Thr</th>
<th>Ile</th>
<th>Leu</th>
<th>His</th>
<th>Phe</th>
<th>Val</th>
<th>Arg</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>4.2</td>
<td>1.9</td>
<td>4.0</td>
<td>4.0</td>
<td>7.6</td>
<td>2.5</td>
<td>5.4</td>
<td>5.9</td>
<td>5.6</td>
<td>41.1</td>
</tr>
<tr>
<td>+ E</td>
<td>4.3</td>
<td>2.0</td>
<td>4.1</td>
<td>4.1</td>
<td>7.7</td>
<td>2.6</td>
<td>5.5</td>
<td>6.0</td>
<td>5.8</td>
<td>42.1</td>
</tr>
</tbody>
</table>

Barley-SBM diet

|        | 13.4    | 3.6     | 9.6     | 11.1    | 18.4    | 6.3     | 12.5    | 12.9    | 16.5    | 104     |
|        | 13.1    | 3.6     | 9.4     | 10.9    | 18.0    | 6.1     | 12.2    | 12.7    | 16.2    | 102     |

Dispensable AA, g/kg DM

<table>
<thead>
<tr>
<th></th>
<th>Ala</th>
<th>Asp</th>
<th>Cys</th>
<th>Glu</th>
<th>Gly</th>
<th>Pro</th>
<th>Ser</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>4.7</td>
<td>6.7</td>
<td>2.8</td>
<td>24.7</td>
<td>4.9</td>
<td>11.2</td>
<td>5.1</td>
<td>60.1</td>
</tr>
<tr>
<td>+ E</td>
<td>4.8</td>
<td>6.8</td>
<td>2.8</td>
<td>25.1</td>
<td>5.0</td>
<td>11.4</td>
<td>5.2</td>
<td>61.1</td>
</tr>
</tbody>
</table>

Barley-SBM diet

|        | 10.8    | 24.2    | 4.6     | 48.1    | 10.7    | 16.6    | 12.7    | 60.0    |
|        | 10.6    | 23.6    | 4.4     | 47.1    | 10.5    | 16.3    | 12.4    | 59.9    |
|        |         |         |         |         |         |         |         |         |

1Rolled barley was dry-stored or high moisture airtight (HMA) stored at 35% moisture without (-E) or with (+E) the combination of phytase, xylanase, β-glucanase, and protease for 49 days, ground and then mixed without (barley diet) or with soybean meal (barley-SBM diet).

2CP was calculated as N (g/kg DM) x 5.45 for the barley diet and as N (g/kg DM) x 6.25 for the barley-SBM diet. 5.45 is a specific nitrogen-to-protein conversion factor for barley (Mariotti et al., 2008).
Table 4. Effect of storage (dry storage vs. HMA storage at 35% moisture) and enzyme combination (without vs. with) on apparent total tract digestibility (ATTD) and apparent ileal digestibility (AID) of N and the balance of N in barley diet (LS means ± SEM)

<table>
<thead>
<tr>
<th>Item</th>
<th>Dry stored barley</th>
<th>HMA stored barley</th>
<th>P-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-E</td>
<td>+E</td>
<td>-E</td>
</tr>
<tr>
<td>DM intake, g/day</td>
<td>1162</td>
<td>1167</td>
<td>1172</td>
</tr>
<tr>
<td>N balance, g/day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intake</td>
<td>21.21</td>
<td>21.58</td>
<td>22.04</td>
</tr>
<tr>
<td>Feces</td>
<td>4.70</td>
<td>3.97</td>
<td>4.02</td>
</tr>
<tr>
<td>Urine</td>
<td>9.19</td>
<td>10.04</td>
<td>9.32</td>
</tr>
<tr>
<td>Net absorption</td>
<td>16.51</td>
<td>17.61</td>
<td>18.02</td>
</tr>
<tr>
<td>Retention</td>
<td>7.32</td>
<td>7.57</td>
<td>8.70</td>
</tr>
<tr>
<td>ATTD of N, %</td>
<td>77.7</td>
<td>81.8</td>
<td>81.7</td>
</tr>
<tr>
<td>AID of N, %</td>
<td>57.8</td>
<td>66.0</td>
<td>60.7</td>
</tr>
<tr>
<td>N utilization, %</td>
<td>33.8</td>
<td>34.7</td>
<td>39.5</td>
</tr>
<tr>
<td>Digestible N, g/kg DM</td>
<td>14.19</td>
<td>15.11</td>
<td>15.37</td>
</tr>
</tbody>
</table>

¹Rolled barley was dry stored or high moisture airtight (HMA) stored at 35% moisture without (-E) or with (+E) the combination of phytase, xylanase, β-glucanase, and protease for 49 days.

²S = 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. Except for AID of N, there was no significant effect of experimental factors on all parameters (P>0.05).

³Utilization, % = (retention/intake) x 100.
Table 5. Effect of storage (dry storage vs. HMA storage at 35% moisture) and enzyme combination (without vs. with) on apparent ileal digestibility (AID, %) of amino acids (AA), % in barley diet (LS means ± SEM)

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary treatment&lt;sup&gt;1&lt;/sup&gt;</th>
<th>P-value&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry stored barley</td>
<td>HMA stored barley</td>
</tr>
<tr>
<td>AID of the indispensable AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>71.9</td>
<td>75.5</td>
</tr>
<tr>
<td>Met</td>
<td>76.6</td>
<td>78.0</td>
</tr>
<tr>
<td>Thr</td>
<td>57.7</td>
<td>60.7</td>
</tr>
<tr>
<td>Ile</td>
<td>68.4</td>
<td>71.7</td>
</tr>
<tr>
<td>Leu</td>
<td>71.8</td>
<td>74.7</td>
</tr>
<tr>
<td>His</td>
<td>71.2</td>
<td>73.7</td>
</tr>
<tr>
<td>Phe</td>
<td>75.1</td>
<td>77.9</td>
</tr>
<tr>
<td>Val</td>
<td>69.4</td>
<td>71.9</td>
</tr>
<tr>
<td>Arg</td>
<td>70.6</td>
<td>74.4</td>
</tr>
<tr>
<td>Average AID</td>
<td>70.2</td>
<td>73.2</td>
</tr>
<tr>
<td>AID of the dispensable AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>57.5</td>
<td>63.6</td>
</tr>
<tr>
<td>Asp</td>
<td>58.6</td>
<td>62.0</td>
</tr>
<tr>
<td>Cys</td>
<td>68.3</td>
<td>73.2</td>
</tr>
<tr>
<td>Glu</td>
<td>79.3</td>
<td>82.9</td>
</tr>
<tr>
<td>Gly</td>
<td>42.8</td>
<td>32.5</td>
</tr>
<tr>
<td>Pro</td>
<td>43.8</td>
<td>69.1</td>
</tr>
<tr>
<td>Ser</td>
<td>63.6</td>
<td>67.0</td>
</tr>
<tr>
<td>Average AID</td>
<td>61.5</td>
<td>70.0</td>
</tr>
<tr>
<td>Total AA AID</td>
<td>65.0</td>
<td>71.3</td>
</tr>
</tbody>
</table>

<sup>1</sup>Rolled barley was dry stored or high moisture airtight (HMA) stored at 35% moisture without (-E) or with (+E) the combination of phytase, xylanase, β-glucanase, and protease for 49 days.

<sup>2</sup>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.
Table 6. The amounts of ileal digestible amino acids (AA) in the barley diets (g digestible/kg DM) (LS means ± SEM)

<table>
<thead>
<tr>
<th>Item</th>
<th>Dry stored barley</th>
<th>HMA stored barley</th>
<th>P-value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-E</td>
<td>+E</td>
<td>-E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indispensable AA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>3.04</td>
<td>3.23</td>
<td>2.97</td>
</tr>
<tr>
<td>Met</td>
<td>1.47</td>
<td>1.52</td>
<td>1.51</td>
</tr>
<tr>
<td>Thr</td>
<td>2.33</td>
<td>2.50</td>
<td>2.30</td>
</tr>
<tr>
<td>Ile</td>
<td>2.74</td>
<td>2.90</td>
<td>2.92</td>
</tr>
<tr>
<td>Leu</td>
<td>5.47</td>
<td>5.78</td>
<td>5.68</td>
</tr>
<tr>
<td>His</td>
<td>1.79</td>
<td>1.89</td>
<td>1.76</td>
</tr>
<tr>
<td>Phe</td>
<td>4.06</td>
<td>4.28</td>
<td>4.29</td>
</tr>
<tr>
<td>Val</td>
<td>4.09</td>
<td>4.31</td>
<td>4.28</td>
</tr>
<tr>
<td>Arg</td>
<td>3.98</td>
<td>4.27</td>
<td>4.26</td>
</tr>
<tr>
<td>Total</td>
<td>29.0</td>
<td>30.7</td>
<td>30.0</td>
</tr>
<tr>
<td>Met+Cys</td>
<td>3.36</td>
<td>3.59</td>
<td>3.29</td>
</tr>
<tr>
<td>Dispensable AA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>2.69</td>
<td>3.03</td>
<td>2.74</td>
</tr>
<tr>
<td>Asp</td>
<td>3.92</td>
<td>4.22</td>
<td>3.76</td>
</tr>
<tr>
<td>Cys</td>
<td>1.89</td>
<td>2.08</td>
<td>1.77</td>
</tr>
<tr>
<td>Glu</td>
<td>19.6</td>
<td>20.8</td>
<td>19.9</td>
</tr>
<tr>
<td>Gly</td>
<td>2.11</td>
<td>1.63</td>
<td>1.83</td>
</tr>
<tr>
<td>Pro</td>
<td>4.93</td>
<td>7.90</td>
<td>7.60</td>
</tr>
<tr>
<td>Ser</td>
<td>3.24</td>
<td>3.49</td>
<td>3.26</td>
</tr>
<tr>
<td>Total</td>
<td>37.0</td>
<td>42.3</td>
<td>40.6</td>
</tr>
<tr>
<td>Grand total AA</td>
<td>66.0</td>
<td>73.0</td>
<td>70.6</td>
</tr>
</tbody>
</table>

¹Rolled barley was dry stored or high moisture airtight (HMA) stored without (-E) or with (+E) the combination of phytase, xylanase, β-glucanase, and protease for 49 days.

²S = storage effect (dry storage vs. HMA storage at 35% moisture). E = enzyme combination effect (without vs. with enzyme combination). The interaction between storage and enzyme combination was insignificant in all parameters (P>0.05).
Table 7. Effect of storage (dry storage vs. HMA storage at 35% moisture) and enzyme combination (without vs. with) on apparent total tract digestibility (ATTD) and apparent ileal digestibility (AID) of N, and the balance of N in barley-soybean meal (barley-SBM) diet (LS means ± SEM)

<table>
<thead>
<tr>
<th>Item</th>
<th>Dry stored barley-SBM</th>
<th>HMA stored barley-SBM</th>
<th>P-value2</th>
<th>SEM</th>
<th>S</th>
<th>E</th>
<th>S x E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Item</td>
<td>-E</td>
<td>+E</td>
<td>-E</td>
<td>+E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM intake, g/day</td>
<td>1223</td>
<td>1233</td>
<td>1177</td>
<td>1189</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N balance, g/day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intake</td>
<td>49.1</td>
<td>46.8</td>
<td>46.6</td>
<td>47.0</td>
<td>0.7</td>
<td>0.104</td>
<td>0.164</td>
</tr>
<tr>
<td>Feces</td>
<td>5.88</td>
<td>5.61</td>
<td>5.87</td>
<td>5.45</td>
<td>0.42</td>
<td>0.829</td>
<td>0.382</td>
</tr>
<tr>
<td>Urine</td>
<td>17.4</td>
<td>16.7</td>
<td>16.2</td>
<td>18.8</td>
<td>0.9</td>
<td>0.627</td>
<td>0.244</td>
</tr>
<tr>
<td>Net absorption</td>
<td>43.2</td>
<td>41.2</td>
<td>40.8</td>
<td>41.6</td>
<td>0.6</td>
<td>0.085</td>
<td>0.302</td>
</tr>
<tr>
<td>Retention</td>
<td>25.7</td>
<td>24.5</td>
<td>24.6</td>
<td>22.8</td>
<td>1.1</td>
<td>0.168</td>
<td>0.135</td>
</tr>
<tr>
<td>ATTD of N, %</td>
<td>88.0</td>
<td>88.0</td>
<td>87.5</td>
<td>88.4</td>
<td>0.8</td>
<td>0.909</td>
<td>0.545</td>
</tr>
<tr>
<td>AID of N, %</td>
<td>75.0</td>
<td>74.5</td>
<td>72.8</td>
<td>72.2</td>
<td>3.31</td>
<td>0.469</td>
<td>0.858</td>
</tr>
<tr>
<td>N utilization, %3</td>
<td>52.4</td>
<td>52.3</td>
<td>52.6</td>
<td>48.4</td>
<td>2.12</td>
<td>0.320</td>
<td>0.249</td>
</tr>
<tr>
<td>Digestible N, g/kg DM</td>
<td>35.3</td>
<td>33.4</td>
<td>34.7</td>
<td>34.9</td>
<td>0.32</td>
<td>0.149</td>
<td>0.013</td>
</tr>
</tbody>
</table>

1Rolled barley was dry stored or high moisture airtight (HMA) stored at 35% moisture without (-E) or with (+E) the combination of phytase, xylanase, β-glucanase, and protease for 49 days, ground and then mixed with soybean meal (SBM).

2S x E = the interaction between storage and enzyme. Except for N absorption and digestible N, no significant effect was observed in other parameters (P>0.05).

3Utilization, % = (Retention/Intake) x 100.
Table 8. Effect of storage (dry storage vs. HMA storage at 35% moisture) and enzyme combination (without vs. with) on apparent ileal digestibility (AID, %) of amino acids (AA) in barley-soybean meal (SBM) diet (LS means ± SEM)

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary treatment</th>
<th>Dry stored barley-SBM</th>
<th>HMA stored barley-SBM</th>
<th>SEM</th>
<th>S</th>
<th>E</th>
<th>S x E</th>
</tr>
</thead>
<tbody>
<tr>
<td>AID of the</td>
<td>-E</td>
<td>+E</td>
<td>-E</td>
<td>+E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indispensable AA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>83.3</td>
<td>82.4</td>
<td>85.4</td>
<td>86.3</td>
<td>1.70</td>
<td>0.105</td>
<td>0.989</td>
</tr>
<tr>
<td>Met</td>
<td>84.4</td>
<td>83.5</td>
<td>84.9</td>
<td>84.5</td>
<td>1.37</td>
<td>0.582</td>
<td>0.646</td>
</tr>
<tr>
<td>Thr</td>
<td>73.2</td>
<td>73.2</td>
<td>70.5</td>
<td>69.8</td>
<td>2.88</td>
<td>0.301</td>
<td>0.917</td>
</tr>
<tr>
<td>Ile</td>
<td>81.7</td>
<td>80.8</td>
<td>79.6</td>
<td>80.3</td>
<td>2.20</td>
<td>0.575</td>
<td>0.957</td>
</tr>
<tr>
<td>Leu</td>
<td>81.7</td>
<td>81.0</td>
<td>79.9</td>
<td>80.7</td>
<td>2.19</td>
<td>0.621</td>
<td>0.973</td>
</tr>
<tr>
<td>His</td>
<td>81.5</td>
<td>80.8</td>
<td>80.2</td>
<td>81.6</td>
<td>1.72</td>
<td>0.908</td>
<td>0.832</td>
</tr>
<tr>
<td>Phe</td>
<td>82.6</td>
<td>81.4</td>
<td>81.1</td>
<td>81.8</td>
<td>2.34</td>
<td>0.814</td>
<td>0.918</td>
</tr>
<tr>
<td>Val</td>
<td>78.9</td>
<td>78.7</td>
<td>77.3</td>
<td>77.1</td>
<td>2.40</td>
<td>0.504</td>
<td>0.927</td>
</tr>
<tr>
<td>Arg</td>
<td>86.3</td>
<td>84.9</td>
<td>86.3</td>
<td>86.9</td>
<td>1.48</td>
<td>0.507</td>
<td>0.757</td>
</tr>
<tr>
<td>Average AID</td>
<td>81.7</td>
<td>80.9</td>
<td>80.7</td>
<td>81.2</td>
<td>1.82</td>
<td>0.874</td>
<td>0.939</td>
</tr>
<tr>
<td>AID of the</td>
<td>+E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dispensable AA</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>75.5</td>
<td>75.2</td>
<td>74.6</td>
<td>74.5</td>
<td>2.69</td>
<td>0.765</td>
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<td>Asp</td>
<td>74.4</td>
<td>74.1</td>
<td>72.4</td>
<td>72.6</td>
<td>3.40</td>
<td>0.609</td>
<td>0.994</td>
</tr>
<tr>
<td>Cys</td>
<td>69.3</td>
<td>67.2</td>
<td>63.8</td>
<td>63.8</td>
<td>4.23</td>
<td>0.305</td>
<td>0.815</td>
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<tr>
<td>Glu</td>
<td>77.8</td>
<td>76.5</td>
<td>74.7</td>
<td>76.6</td>
<td>3.75</td>
<td>0.698</td>
<td>0.927</td>
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<tr>
<td>Gly</td>
<td>58.3</td>
<td>60.1</td>
<td>57.2</td>
<td>59.4</td>
<td>4.01</td>
<td>0.815</td>
<td>0.622</td>
</tr>
<tr>
<td>Pro</td>
<td>77.6</td>
<td>74.8</td>
<td>76.5</td>
<td>77.4</td>
<td>2.64</td>
<td>0.792</td>
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<tr>
<td>Ser</td>
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<td>77.0</td>
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<td>75.5</td>
<td>2.59</td>
<td>0.587</td>
<td>0.963</td>
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<tr>
<td>Average AID</td>
<td>74.9</td>
<td>74.1</td>
<td>72.7</td>
<td>73.7</td>
<td>2.94</td>
<td>0.697</td>
<td>0.974</td>
</tr>
<tr>
<td>Total AA AID</td>
<td>78.0</td>
<td>77.2</td>
<td>76.3</td>
<td>77.1</td>
<td>2.41</td>
<td>0.758</td>
<td>0.998</td>
</tr>
</tbody>
</table>

1Rolled barley was dry stored or high moisture airtight (HMA) stored without (-E) or with (+E) the combination of phytase, xylanase, β-glucanase, and protease for 49 days, ground and then mixed with soybean meal (SBM).

There was no significant effect of storage, enzyme combination and their interaction in all parameters (P>0.05).
Table 9. The amounts of ileal digestible amino acids (AA) in the barley-SBM diets (g digestible/kg DM) (LS means ± SEM)

<table>
<thead>
<tr>
<th>Item</th>
<th>Dietary treatment¹</th>
<th></th>
<th>SEM</th>
<th>P-value²</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry stored barley-SBM &amp; HMA stored barley-SBM</td>
<td>-E</td>
<td>+E</td>
<td>-E</td>
<td>+E</td>
<td>S</td>
<td>E</td>
<td>S x E</td>
</tr>
<tr>
<td>Indispensable AA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>11.2 10.8 11.6 11.9</td>
<td>0.23</td>
<td></td>
<td>0.007</td>
<td>0.965</td>
<td>0.119</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>3.05 2.98 3.06 3.11</td>
<td>0.05</td>
<td></td>
<td>0.202</td>
<td>0.861</td>
<td>0.226</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>7.02 6.88 6.81 6.89</td>
<td>0.28</td>
<td></td>
<td>0.714</td>
<td>0.918</td>
<td>0.697</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>9.06 8.81 8.94 9.23</td>
<td>0.25</td>
<td></td>
<td>0.564</td>
<td>0.933</td>
<td>0.294</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>15.0 14.6 14.8 15.3</td>
<td>0.41</td>
<td></td>
<td>0.560</td>
<td>0.929</td>
<td>0.303</td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>5.10 4.96 5.01 5.21</td>
<td>0.12</td>
<td></td>
<td>0.498</td>
<td>0.804</td>
<td>0.498</td>
<td></td>
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</tr>
<tr>
<td>Phe</td>
<td>10.3 10.0 10.2 10.5</td>
<td>0.26</td>
<td></td>
<td>0.476</td>
<td>0.950</td>
<td>0.245</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>10.2 10.0 10.1 10.2</td>
<td>0.32</td>
<td></td>
<td>0.800</td>
<td>0.968</td>
<td>0.550</td>
<td></td>
<td></td>
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<tr>
<td>Agr</td>
<td>14.2 13.7 14.4 14.8</td>
<td>0.25</td>
<td></td>
<td>0.027</td>
<td>0.798</td>
<td>0.086</td>
<td></td>
<td></td>
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<tr>
<td>Total</td>
<td>85.1 82.7 84.8 82.7</td>
<td>1.94</td>
<td></td>
<td>0.343</td>
<td>0.989</td>
<td>0.275</td>
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<tr>
<td>Met+Cys</td>
<td>6.14 5.91 5.86 5.96</td>
<td>0.23</td>
<td></td>
<td>0.639</td>
<td>0.807</td>
<td>0.493</td>
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<tr>
<td>Dispensable AA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>8.16 7.94 8.12 8.26</td>
<td>0.27</td>
<td></td>
<td>0.642</td>
<td>0.910</td>
<td>0.547</td>
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<tr>
<td>Asp</td>
<td>18.0 17.5 17.6 18.2</td>
<td>0.77</td>
<td></td>
<td>0.882</td>
<td>0.992</td>
<td>0.549</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>3.08 2.93 2.80 2.85</td>
<td>0.17</td>
<td></td>
<td>0.353</td>
<td>0.796</td>
<td>0.598</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>37.4 36.0 35.4 36.9</td>
<td>1.64</td>
<td></td>
<td>0.760</td>
<td>0.964</td>
<td>0.443</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>6.26 6.31 6.21 6.57</td>
<td>0.40</td>
<td></td>
<td>0.821</td>
<td>0.641</td>
<td>0.729</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>12.9 12.2 12.7 13.0</td>
<td>0.40</td>
<td></td>
<td>0.497</td>
<td>0.739</td>
<td>0.288</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>9.77 9.56 9.69 9.86</td>
<td>0.31</td>
<td></td>
<td>0.747</td>
<td>0.947</td>
<td>0.592</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>95.5 92.5 92.5 95.6</td>
<td>3.77</td>
<td></td>
<td>0.992</td>
<td>0.992</td>
<td>0.474</td>
<td></td>
<td></td>
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<tr>
<td>Grand total AA</td>
<td></td>
<td>181</td>
<td>175</td>
<td>177</td>
<td>183</td>
<td>5.65</td>
<td>0.736</td>
<td>0.998</td>
</tr>
</tbody>
</table>

¹Rolled barley was dry stored or high moisture airtight (HMA) stored at 35% moisture without (-E) or with (+E) the combination of phytase, xylanase, β-glucanase, and protease for 49 days, ground and then mixed with soybean meal (SBM).

²S = storage effect (dry storage vs. HMA storage at 35% moisture). E = enzyme combination effect (without vs. with enzyme combination. The interaction between storage and enzyme combination is insignificant in all parameters (P>0.05). Except for Lys and Arg, the main effects of storage and enzyme combination are insignificant in all parameters (P>0.05).
Chapter VI. **Discussion**

1. **Factors influencing nutrient solubility of cereals during high moisture airtight storage**

   In this chapter, the effects of grain processing, grain moisture and storage time on the nutrient solubility are discussed based on the results of the treatments without the enzyme combination. The effect of the enzyme combination is discussed in a separate section together with cereal type and the interaction between the enzyme combination and other factors.

1.1. **Grain moisture**

   The normal procedure for storing cereals (dry storage) requires grain moisture equal to or below 13%, so grain moisture is one of the safety criteria to maintain the grain quality (Laca et al., 2006). The low grain moisture level inhibits the growth of microorganisms, especially moulds, and minimises the loss of nutrients during storage. HMA storage preserves cereals at a high moisture level, and the airtight condition inhibits the decomposition caused by the aerobic microorganisms.

   The moist condition is required to activate the endogenous enzymes in cereals and to promote the microbial fermentation during HMA storage. The current study confirmed grain moisture as the most important factor that influences the solubilisation of nutrients during HMA storage. At 20 and 23% moisture, HMA storage of barley and triticale led to no change or a limited enhancement in the phytate degradation and the solubility of P and N compared with dry storage (Manuscript I). However, increasing the grain moisture from 20% to 29% linearly increased the phytate degradation (as expressed in Phytate P:Total P) (in barley and triticale) and the solubility of P (in barley and triticale) and N (in triticale) and (Manuscript I). The positive linear relationship indicates that grain moisture is one of the limiting factors of the study, and a higher moisture level (above 29%) may increase the phytate reduction the improvement of P and N solubility (Manuscript I). This was proved in Manuscript II, as HMA stored barley at 35% and 40% moisture results in a higher solubility of P, N and protein and an almost complete degradation of phytate compared with a 29% moisture. Likewise, Åman et al. (1990a) found an increase in the solubility of N (13% points) and starch (10% points) and a rapid decrease in β-glucan solubility (40% points) in HMA stored barley at 36% moisture, whereas little or no change in nutrient solubility was observed in HMA stored barley at 23% and 25% moisture. Baron et al. (1986) also stated
that HMA stored maize at 22% and 26% moisture contained only half the soluble N content in HMA stored barley at 33 and 36% moisture.

The increased grain moisture content enhances the proteolytic activities of endogenous enzymes and microbial fermentation (Buchanan-Smith et al., 2003; Makoni et al., 1997; Rooke and Hatfield, 2003). Microbial fermentation is associated with the production of lactic acid, and thereby the pH of HMA stored cereals may drop below 5 (Baron et al., 1986). The magnitude of the effect of endogenous enzymes and microbial fermentation on the enhancement in the nutrient value of HMA stored cereals depends on the grain moisture content and the level of endogenous enzyme activity in cereals. For example, a drop of pH below 5 was only observed in the HMA stored maize at 33% and 36% moisture (Baron et al., 1986) and in the HMA stored barley at 40% moisture (Manuscript II). This implies that microbial fermentation to some extent may have occurred in these groups. However, the observed pH in HMA stored maize at 22% and 26% moisture (6.0-5.5) (Baron et al., 1986), in HMA stored triticale at 20% to 29% moisture (6.7-5.7) (Manuscript I) and in HMA stored barley at moisture levels up to 35% (6.2-5.7) (Manuscripts I and II) indicated that fermentation occurred to a very limited extent. It seems that the endogenous enzymes in cereals were mainly responsible for the enhancement in nutrient solubility in barley (up to 35% moisture) and in triticale (up to 29% moisture), whereas fermentation may play a role at higher moisture levels (above 35% in barley) during HMA storage. Maize, however, possesses a negligible phytase activity, so the phytate degradation in HMA stored maize was mainly due to lactic acid fermentation that results in a pH drop to below 5 and a greater lactic acid concentration (Baron et al., 1986; Humer et al., 2013).

The current study observed a negative linear correlation between phytase activity and moisture levels in HMA stored rolled barley at day 49 (Manuscript I). Thus, dry stored barley and triticale contained a higher phytase activity compared to HMA stored barley and triticale after 49 days of storage (Manuscripts I and II). These results imply that:

1. The endogenous phytase in barley and triticale is not activated during dry storage and requires certain moisture levels (above 20% moisture) to be activated in HMA stored barley and triticale. Thus, no change in P solubility and phytate degradation was observed in dry stored and HMA stored barley and triticale at 20% moisture.
(2) Increasing the grain moisture content may enhance the activation and activity of endogenous phytase in barley and triticale on phytate degradation to release P during HMA storage and consequently leads to a higher P solubility.

(3) The activity of endogenous phytase in barley and triticale was decreased during HMA storage.

The current results agreed with other studies that observed the decrease in endogenous phytase activity in cereals after soaking for 24 hours (Blaabjerg et al., 2010a) and 48 hours (Carlson and Poulsen, 2003). The same trend may occur for other endogenous enzymes, so the activity of endogenous β-glucanase in HMA stored barley was also decreased from 5900 BU/kg DM (before storage) to 3120 BU/kg DM at day 49 (Manuscript II).

The endopeptidases in cereals initiate the protein degradation by cleaving the internal peptide bonds in the polypeptide chain (Rooke and Hatfield, 2003). The end products of proteolytic enzyme activities normally includes free amino acids and peptides, whereas the further degradation to ammonia and amines are caused by the microbial fermentation rather than the endogenous enzymes (Rooke and Hatfield, 2003). Similarly, the microbial decarboxylation that takes place in fermented liquid feeding also transforms free amino acids of cereals, especially Lys, to biogenic amines and results in the irreversible amino acid loss (Canibe and Jensen, 2010). Another point is that a rapid decline in pH due to lactic acid production may be unfavourable to the activities of endogenous phytase in cereals and microbial phytases produced by lactic acid bacteria on phytate degradation (Humer et al., 2013). Therefore, it may be better to enhance the phytate degradation and the nutrient solubility, especially the N and protein solubility, by the endogenous enzymes in cereals during HMA storage to overcome the drawbacks of fermentation. In the current study, HMA storage of barley at 40% moisture showed the greatest nutrient solubility and the lowest (negligible) amounts of remaining phytate. However, 35% moisture seems to be the optimum moisture level for HMA storage of barley to prevent the loss of energy and N via ammonia by microbial fermentation which was nil or very low during HMA storage of barley at 35% moisture.

The current study used the same barley cultivar (Zephyr) for all experiments. However, the solubility of P and N of HMA stored barley at 29% in Experiment 1 (0.38 and 0.30, respectively; Manuscript I) was higher compared with that in Experiment 2 (0.34 and 0.21, respectively; Manuscript II). This is most likely due to a higher storage temperature in
Experiment 1 (15-18°C) compared with Experiment 2 (15°C). Likewise, Carlson and Poulsen (2003) observed that soaking of barley at 20°C led to a faster degradation rate of phytate compared with that at 10°C. The same temperature depending effect was observed in Experiment 2 (15°C) compared with the results obtained at a lower HMA storage temperature (9°C) in Experiment 3. In Experiment 3, barley was dry stored or stored at 35% moisture in airtight bags (400 kg/bag) at an ambient temperature in the barn (without heating system) for 49 days. The average temperature in this period was 9°C, but the outdoor temperature fluctuated in a broad range from -2 to 22°C. HMA storage of barley at 35% moisture enhanced the nutrient solubility and phytate degradation to a greater extent in Experiment 2 compared with those in Experiment 3 (Manuscripts II and III). The outdoor temperature fluctuation observed in Experiment 3 may delay the activation or affect the activity of endogenous enzymes in barley and thus reduce the degradation rate of phytate and NSP. Therefore, the storage temperature could be as important as the grain moisture level during HMA storage, because the increased temperature may also enhance the activities of endogenous enzymes and microorganisms that boost the degradation and fermentation processes.

1.2. Grain processing before storage

The biological activities (e.g. germination) control the synthesis and the release of endogenous enzymes in the intact whole grains (Sauer, 1992). Milling, however, destroys the biological order of the grains, increases the water adsorption capacity and reactivity and facilitates and enhances the reactions to external factors such as oxygen, light and moisture during storage (Sauer, 1992). The current study observed that the effect of grain processing before storage (whole grain vs. rolled grain) on the solubility of P and N of the HMA stored cereals closely relates to the grain moisture content. At moisture levels below 26%, HMA storage of barley and triticale had a limited effect on the P and N solubility, so the difference between whole and rolled grains was negligible (Figures 1-4, Manuscript I). At 26% and 29% moisture, rolling of barley and triticale before storage enhanced the solubility of P and N of HMA stored barley and triticale compared with no processing (whole barley and whole triticale) (Figures 1-4, Manuscript I). The current findings support previous observations that HMA stored ground maize at 26% and 33% moisture resulted in a higher N solubility (7% and 14% points) compared with HMA stored whole maize (Baron et al., 1986). This is most likely because grinding or rolling disrupts the grain matrix, exposes the substrates (P and N) to the enzymatic and chemical degradation and consequently
enhances the degradation rate of microbial or endogenous enzymes (Baron et al., 1986; Buchanan-Smith et al., 2003; Prigge et al., 1976). Grinding also increased the production of lactic acid resulting in a decline in pH to a higher degree in HMA stored maize at 33 and 36% moisture (Baron et al., 1986). Baron et al. (1986) observed a higher production rate but a similar total amount of soluble N at day 90 in HMA stored ground maize compared with that in HMA stored whole maize at 36% moisture. It implies that at a grain moisture content higher than 30%, the slower degradation rate in whole maize may compensate by extending the storage time.

### 1.3. Storage time

In the current study, barley and triticale were stored in airtight conditions for 14, 29 and 49 days. The effect of storage time on the phytate degradation and the solubility of P and N of HMA stored barley and triticale was influenced by grain moisture and grain processing (Manuscript I). In HMA stored rolled barley, a positive linear relationship was observed between storage time and P solubility (20%, 23%, 26% and 29% moisture) or N solubility (29% moisture) (Manuscript I). Thus, extending the storage time enhanced the P and N solubility in HMA stored rolled barley, but the rate of increase seemed to be more rapid during the first 14 days compared with from 14 to 49 days (Manuscript I). HMA storage had no effect on enhancing the P and N solubility of whole barley, the N solubility of rolled barley (except at 29% moisture), whole triticale and rolled triticale (except at 26% and 29% moisture) even after storing for 49 days (Manuscript I). However, the P solubility reached a plateau state at day 14 or day 29 in HMA stored whole and rolled triticale at all moisture levels (except rolled triticale at 23% moisture), and the N solubility reached a plateau state at day 29 in HMA stored rolled triticale at 26% and 29% moisture.

The reported effect of storage time on nutrient solubility in previous studies is inconsistent. The N solubility of HMA stored whole barley at 35% moisture increased during 70 days of storage, remained unchanged during winter and continued to enhance in summer after 1 year of storage (Åman et al., 1990a). However, the N solubility only increased slightly in HMA stored whole barley at 23 and 25% moisture after storing for 1 year (Åman et al., 1990a). Likewise, Baron et al. (1986) also observed that the increase in soluble N reached a plateau state at day 15 in HMA stored maize at 22% and 26% moisture, whereas the N solubility continued to increase in HMA stored maize at 33% and 36% moisture even after storage for 90 days. The effect of storage time on nutrient solubility was not evaluated in HMA stored barley at 35% and 40% moisture in the current
study. However, it seems that extending the storage time will enhance nutrient solubility and phytate degradation to a greater extent in HMA stored cereals with the moisture content above 30% compared with the lower moisture content. Moreover, the proteolysis and phytate degradation rate during HMA storage was slower in whole cereals compared with rolled cereals. Thus, HMA storage of whole cereals may require a longer time (e.g. 90 days) to achieve the same level of nutrient solubility in HMA stored ground or rolled cereals at, e.g. day 30.

1.4. Cereal type and enzyme combination

In the current study, triticale showed a higher N solubility (0.31 vs. 0.17) and Phytate P:Total P ratio (0.79 vs. 0.71) but a similar P solubility (0.21 vs. 0.19) compared with barley (Manuscript I). These results are in accordance with Christensen (2013b) who also observed a higher soluble protein in triticale (23%) compared with barley (17%). Christensen (2013b) observed a higher increase in soluble protein in triticale (21% points) compared with barley (10% points) after soaking for 72 hours. Similarly, HMA storage at 29% moisture enhanced the solubility of P and N to a greater extent in rolled triticale (31% and 15% points, respectively) compared with rolled barley (16% and 11% points, respectively) at day 49 (Manuscript I). This was in agreement with the observed greater phytate degradation in triticale (26% points) compared with barley (13% points) after HMA storage for 49 days (Manuscript I).

The current study also revealed that the change in nutrient solubility during HMA storage differed between barley and triticale. While P solubility increased linearly over storage time in HMA stored barley, it increased more rapidly during the first 15 days and reached a plateau state at day 15 or day 30 in HMA stored triticale (Manuscript I). Similarly, a faster increase in soluble protein during 8 hours of soaking was observed in triticale compared with barley (Christensen, 2013b). This may be explained by the higher endogenous enzyme activity in triticale in comparison with barley (1130 vs. 426 FTU phytase/kg DM; 204 vs. <100 U xylanase/kg DM; and 49400 vs. 5930 BU β-glucanase/kg DM, Manuscript I). Also, triticale exhibits a greater cysteine endoproteases activity and a higher overall proteolytic activity compared with barley and other cereals (Jones and Lookhart, 2005).

The differences in grain morphology and endogenous enzyme activities between barley and triticale may also contribute to the different responses to microbial enzyme supplementation. A lower P solubility was observed in barley with no enzyme addition
(solely endogenous enzymes) compared with barley added the enzyme combination (endogenous enzymes plus added enzymes) regardless of the storage method and time (Manuscript I). It is most likely because the enzymes may have been activated and increased the P solubility during the supernatant extraction process where barley, enzymes and water were mixed together for 10 minutes. This is similar to the initial rapid phytate hydrolysis rate observed in previous studies, when soaking microbial phytase with wheat, soybean meal and rapeseed cake (Blaabjerg et al., 2010a), maize (Niven et al., 2007) and rapeseed meal (Newkirk and Classen, 1998). To minimise this activity of added microbial enzymes at time 0, Jakobsen (2015) suggested to add the enzymes immediately before sampling and immerse the samples in liquid nitrogen immediately to eliminate the lag-time during sampling and freezing. However, this fast effect of the enzymes during extraction may also occur when mixing with feed digesta in the pigs’ stomach and can be considered a part of the overall enzyme effect. Interestingly, this fast response of the enzyme combination was seen in P solubility but not in N solubility and Phytate P:Total P in barley (but not in triticale). In fact, the enhancing effect of the enzyme combination on the nutrient solubility seemed to be more consistent in triticale compared with barley. The enzyme combination enhanced the N solubility and the P solubility at the same time (Manuscript I). However, the increase in P solubility in barley due to the enzyme combination did not result in a corresponding increase in N solubility. Consequently, increasing P solubility in the presence of the enzyme combination led to a linear increase in N solubility but to a lower extent (6% points on average) compared with no enzyme addition (Manuscript I). This could be explained by differences in grain morphology, as the P pool in barley is more readily accessible for the enzymes compared with triticale. The high endogenous enzyme activity in triticale may also limit the responses to microbial enzymes in triticale as observed in previous studies (Blaabjerg et al., 2010a; Rodehutscord et al., 1996). Substrate preference may be another aspect, so the enzyme combination may have a higher affinity to the P-substrates compared with the N-substrates. In general, both endogenous enzymes in cereals and the enzyme combination are more effective in increasing the P solubility of barley and triticale than the N solubility. This may be due to the unfavourable conditions for proteolytic enzymes during HMA storage. The pH of HMA stored barley was in the range from 6.2 to 5.7, whereas the optimum pH of proteolytic endogenous enzymes in barley was above 7 for serine protease (Delcour and Hoseney, 2010) and metalloproteases (Jones, 2005) and acidic pH (3.5-4.8) for cysteine protease.
The added microbial protease used in the current study is also a serine protease with the chemotrypsin specificity from *Nocardiopsis prasina* expressed in *Bacillus licheniformis*, which most likely will have a pH optimum above 7. Thus, adapting the storage conditions to the optimum conditions for endogenous enzymes in cereals and added microbial enzymes may enhance the positive effect of HMA storage and the enzyme combination even further.

Moreover, except for the increase in P solubility of barley at all moisture levels, the effect of the enzyme combination on enhancing the solubility of P and N was only observed in rolled barley and rolled triticale at 26% and 29% moisture. This proved that the effect of the enzyme combination on nutrient solubility also depended on the grain moisture content and the grain processing. Similarly with the endogenous enzymes in cereals, the added microbial enzymes enhanced their efficacy in a high moisture condition (at least 26% moisture) with a high substrate accessibility (rolled or ground grains).

As mentioned in the previous section, the mechanisms behind the effect of HMA storage on enhancing the phytate degradation and the solubility of P and N seems to be different between various cereals depending on their endogenous enzyme activity. In cereals showing high endogenous enzyme activity like barley and triticale, the increase in phytate degradation and the solubility of P and N during HMA storage seems to be mainly caused by the endogenous enzymes. However, lactic acid fermentation plays a vital role in proteolysis and hydrolysis of phytate in HMA stored cereals with a limited endogenous enzyme activity like maize which results in a low pH (at least below 5) and a high lactic acid concentration (Humer et al., 2013).

2. Effect of storage and enzyme combination on nutrient digestibility in pigs

2.1. Phosphorus

HMA storage of barley at 35% moisture enhanced the ATTD of P in barley by 15% points (Manuscript II). This is most likely due to the increase in P solubility and phytate degradation by the endogenous enzymes in HMA stored barley before feeding (Manuscript II). Moreover, the degradation of phytate may make P more accessible and more digestible to enzymes in the digestive tract of pigs.

Research evaluating the effects of HMA storage on P digestibility in barley is limited and inconsistent. Weltzien and Aherne (1986) observed an increase in AID of P in HMA stored ground barley at 29% moisture (15% points) compared with dry stored ground
barley. However, the AID of P in HMA stored rolled barley at 29% moisture was similar to dry stored rolled barley (Weltzien and Aherne, 1986). HMA storage of barley at 25% and 35% moisture also increased the ATTD of P numerically (20% and 13% points, respectively) but insignificant (Pieper et al., 2011). Unfortunately, these 2 studies did not analyse the phytate content and phytase activity in the diets, so, it is difficult to explain the results. However, several factors such as barley cultivar, animal ages and methodology may influence the results of these studies. In the studies of Weltzien and Aherne (1986) and Pieper et al. (2011), the supplementation of inorganic phosphates and a high variation of P digestibly may also have blurred the effect of HMA storage.

In agreement with the current study, the positive effect of HMA storage on enhancing the ATTD of P was also observed in triticale (32% points) and wheat (22% points) at 25% moisture (Pieper et al., 2011); and in maize at 23% and 25% moisture (14% and 9% points, respectively) (Humer et al., 2013). Even though the grain moisture content was lower (<30%) compared with the current study (35%), the increase in ATTD of P due to HMA storage was highest in triticale and wheat (Pieper et al., 2011), intermediate in barley (Manuscript II) and lowest in maize (Humer et al., 2013). Most likely this is due to the endogenous phytase activity was highest in triticale (840-2039 FTU/kg) and wheat (300-2000 FTU/kg), intermediate in barley (200-882 FTU/kg) and negligible in maize (<100 FTU/kg) (Weremko et al., 1997).

Interestingly, HMA storage of triticale and wheat at 35% moisture (same level as in the current study) failed to increase the ATTD of P compared with dry storage in the study of Pieper et al. (2011). This is most likely due to the supplement of lactic acid bacteria (Lactobacillus plantarum) that modified the storage condition during HMA storage of cereals in the study of Pieper et al. (2011). With lactic acid bacteria supplementation, HMA stored cereals at 35% moisture enhanced the lactic acid fermentation that resulted in the production of lactic acid and declined pH to a greater extent than HMA storage at 25% moisture (Pieper et al., 2011). As discussed in section 1.1 of Chapter VI, the excessive lactic acid concentration and the rapid drop in pH may reduce the efficacy of endogenous phytase in cereals and microbial phytase which may explain the lack of effect of HMA storage on the ATTD of P in triticale and wheat at 35% moisture (Humer et al., 2013).

Enzymes require moist condition to be activated and sufficient time for the enzymes to degrade substrates. Thus, the short retention time for phytate degradation along the digestive tract (average 5-6 hours) together with the low pH (below 4) in the stomach may
limit the efficacy of enzymes, especially the endogenous enzymes in cereals, in improving the ATTD of P (Blaabjerg et al., 2011). The endogenous phytase activity declines rapidly at pH below 4, whereas the microbial phytases remain active even at pH below 3 (Konietzny and Greiner, 2002). Adding the enzyme combination to dry stored barley (endogenous enzymes plus added enzyme) enhanced the ATTD of P (18% points) to the same extent as HMA stored barley at 35% moisture (20% points) (solely endogenous enzymes) for 49 days (Manuscript II). This proved that the short retention time along the digestive tract is one of the limiting factors that hinder a higher increase in the ATTD of P in pigs. Moreover, it is possible to use the endogenous enzymes in cereals to enhance the ATTD of P if pH is proper and a sufficient time for the enzymes’ active site to access substrates is given. The inclusion of the enzyme combination to HMA stored barley at 35% moisture for 49 days provided the endogenous enzymes in barley as well as the added microbial enzymes more time to degrade their substrates resulting in the greatest enhancement in ATTD of P in pigs (70%, Manuscript II).

According to the improvement in ATTD of P, HMA storage of barley and the enzyme combination also reduced the excretion of P in faeces (29% and 34%, respectively) but at the same time increased the urinary P excretion (26% and 35%, respectively) (Manuscript II). In another words, the HMA storage of barley and the enzyme combination shifted the P excretion route from faeces to urine. This increased urinary P excretion is most likely due to the extremely low level of daily Ca intake (< 1g/day) – solely provided from barley with or without SBM – that did not fulfill the pig’s requirement which resulted in the current negative Ca absorption, retention and digestibility (Manuscript II). The low dietary Ca stimulates the release of parathyroid hormone (PTH) (Brown, 1991) and consequently decreases the reabsorption of renal P and increases the excretion of P in urine (Klein, 2013). Thus, as long as the metabolic Ca and P for bone mineralisation is imbalance, the increase in absorbed P cannot be retained but be excreted via urine as in the current study. It is speculated that the 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 maximise P digestibility and support maximum P retention.

The endogenous phytase activity in cereals like wheat and barley may also increase the phytate degradation in protein feedstuffs like SBM that normally have negligible phytase activity (Fandrejewski et al., 1997). HMA storage of barley enhanced the ATTD of P to a lower extent in barley-SBM diet compared with barley diet (6% vs. 15%, respectively)
PhD thesis - Discussion

(Manuscript II). The calculated ATTD of P of SBM in diets based on the dry stored barley was higher than in diets based on the HMA stored barley (Manuscript II). This is most likely because of the lower enzyme activity in the HMA stored barley than in the dry stored barley when fed to pigs together with SBM (Manuscript II). Moreover, the addition of the enzyme combination had no further effect on the calculated ATTD of P of SBM in diets based on the dry stored barley (Manuscript II). This shows that the endogenous enzymes in barley resulted in the same ATTD of P of SBM (calculated) as with the addition of the enzyme combination in diets based on the dry stored barley. However, adding the enzyme combination enhanced the ATTD of P of SBM (calculated) numerically (12% points) in diets based on the HMA stored barley (Manuscript II). This is most likely due to the decrease in endogenous enzyme activity in HMA stored barley after storage (Manuscript II). Therefore, it seems to be necessary to supply microbial enzymes to enhance the ATTD of P of SBM when fed together with HMA stored barley to compensate for the decrease in endogenous enzymes after storage.

2.2. Nitrogen and amino acids

The current study observed no effect of HMA storage of barley at 35% moisture on enhancing the ATTD of N in barley diets and barley-SBM diets (Manuscript III). Similar results were also found in previous studies with HMA stored barley at 25% and 35% moisture (Pieper et al., 2011), 29% moisture (Weltzien and Aherne, 1987) and 30% moisture (Hackl et al., 2010); HMA stored wheat at 30% (Hackl et al., 2010) and 35% moisture (Pieper et al., 2011); HMA stored triticale at 25% and 35% moisture (Pieper et al., 2011) and 30% moisture (Hackl et al., 2010); and HMA stored maize at 30% moisture (Humer et al., 2013). However, an increase in the ATTD of N was observed in pigs fed HMA stored barley-wheat at 18% moisture (3% points, P=0.08) (Poulsen et al., 2012) and HMA stored wheat at 25% moisture (2% points, P<0.05) (Pieper et al., 2011). The inconsistent results of HMA storage on the ATTD of N may relate to the difference in dietary fibre composition between cereals. Microbial enzymes secreted by the bacteria colonising the distal segment of the small intestines and the large intestine can easily degrade the high soluble NSP fraction (β-glucan) which is present at a higher concentration in barley compared with wheat (Bach Knudsen and Hansen, 1991). Thus, the degradation of NSP in barley by the microorganisms in the small intestine and the large intestine may enhance the release of protein from the grain matrix and thereby conceal the increasing effects on N digestibility of HMA storage of barley and enzyme combination in pigs. As mentioned in the previous
section, the supplementation of lactic acid bacteria in the studies of Hackl et al. (2010) and Pieper et al. (2011) may change the storage conditions during HMA storage to promote the microbial fermentation. The addition of lactic acid bacteria significantly increased the ammonia concentration two-fold in HMA stored triticale and wheat at 35% moisture compared with no lactic acid supplementation (Pieper et al., 2011). With the lactic acid bacteria supplementation, the ammonia loss was also significantly higher in HMA stored wheat at 35% moisture compared with that at 25% moisture (1.41 vs. 0.65 g/kg feed, respectively) (Pieper et al., 2011). Thus, the absence of effects of HMA storage in the ATTD of N in wheat at 30% (Hackl et al., 2010) and 35% moisture (Pieper et al., 2011), but significant increase in the ATTD of N in HMA stored wheat at 25% moisture (Pieper et al., 2011) may be due to the greater extent of fermentation in wheat at 30% and 35% moisture.

Unfortunately, little information is available on the effect of HMA storage on the AID of N of cereals. In line with the study of Weltzien and Aherne (1987) on HMA stored barley at 29% moisture, HMA storage of barley at 35% moisture also had no effect on the AID of N in barley diets and barley-SBM diets (Manuscript III). Nevertheless, HMA storage of barley enhanced the AID of several indispensable amino acids: Met, Arg and Phe (4-5% points) in barley at 35% moisture (Manuscript III); Lys, Met, Ile and Val (5-7% points) in barley at 29% moisture (Weltzien and Aherne, 1987) and the standardised pre-caecal digestibility of Lys, Met, Thr, Leu, Phe and Arg (3-11%) in wheat at 30% moisture (Hackl et al., 2010). Hackl et al. (2010) observed, however, a decrease in AID of Lys and His (6-7% points) in HMA stored barley at 30% moisture. Several factors such as grain moisture content, storage time, cereal type and cultivar may contribute to this inconsistent effect of HMA storage on the AID of amino acids.

The cell walls of barley aleurone layer and endosperm are mainly composed of NSP (arabinoxylan and β-glucan) (Bedford, 1995; Lafiandra et al., 2014). This rigid NSP matrix encapsulates protein, starch and minerals and creates a physical barrier that prevents the digestion and absorption of these essential nutrients (Nortey et al., 2007a). Moreover, phytate also forms insoluble complexes with protein that reduce the digestion and absorption of protein and amino acids (Selle and Ravindran, 2008). Consequently, cereal protein and amino acids are poorly digestible because they are tightly embedded in the crystalline matrix of phytate-protein and protein-NSP (Becraft, 2007). According to Rybka et al. (1992), NSP encapsulated 26-28% total N, 40% Lys, 36% Thr and 33% Met+Cys in
Thus, the enhanced AID of the indispensable amino acids in the current study means that the endogenous enzymes in barley and the added enzyme combination of phytase, xylanase, β-glucanase and protease may have hydrolysed NSP and phytate to release the encapsulated amino acids (Manuscript III).

Limited information is available on the effect of combining various enzymes on the ATTD and AID of N and the AID of amino acid of cereals in pigs. The inclusion of protease and xylanase in combination or individually had no effect on the ATTD and AID of N in pigs fed barley-wheat diets (Mc Alpine et al., 2012; O’Shea et al., 2014). A combination of phytase, xylanase and β-glucanase enhanced the SID and AID of indispensable amino acids to the same extent as an addition of solely phytase but showed its synergistic effect on enhancing the SID of Met in a wheat-barley diet (Kiarie et al., 2010) and the AID of His in a wheat diet (Nortey et al., 2007b). The current inclusion of phytase, xylanase, β-glucanase and protease significantly enhanced the AID of N (7% points), average amino acids (6% points) and Lys (7% points) and tended to increase the AID of Ile, Leu and Phe (4-5% points) in barley (Manuscript III). A combination of xylanase, β-glucanase and protease also enhanced the AID of N (8% points), total NSP (14% points) and all indispensable amino acids (9% points on average) in barley but had no extra effect compared with the addition of xylanase or β-glucanase individually (Yin et al., 2001). A literature review reported that the effect of phytase on the AID of amino acids are contradictory, but the increase in AID of amino acids by microbial phytase may be from 6% to 7% in pigs (Adeola and Cowieson, 2011). The AID of Cys, Thr, Ser, Pro and Gly was increased by the addition of microbial phytase, whereas the response in AID of Met, Arg, Glu and Lys was less (Adeola and Cowieson, 2011). It seems that several factors such as enzyme efficacy, substrate preference, cereal type/cultivar, diet composition and age of the pigs varied among studies that consequently led to the inconsistent results. Yin et al. (2001) also claimed that the response of different barley varieties to the enzyme combination on enhancing the AID of N and amino acids closely related to their dietary fibre content, especially β-glucan. Thus, the addition of microbial enzymes is expected to enhance the nutritive value of barley to a greater extent in barley varieties containing a higher content of β-glucan (Yin et al., 2001). Moreover, it is important to understand the mode of action of the enzymes individually and in combination to maximise their efficacy on enhancing the nutritive value of cereals (Adeola and Cowieson, 2011). The current study is the first experiment demonstrated an effect of adding phytase, xylanase, β-glucanase and protease in
combination on enhancing the AID of N and amino acids of barley in growing pigs. Moreover, the addition of the enzyme combination to HMA stored barley during storage resulted in the greatest improvement in AID of the indispensable amino acids (Lys, Met and Arg, 10-15%) and the amounts of digestible indispensable amino acids (Lys, Met+Cys, Ile and Phe, 7-17%) compared with dry stored barley in barley diets. To maximise this effect, further studies are required to identify the effect of single enzymes and their additivity or interaction in various combinations.

Lys, Met, Thr, Trp and Val are the commercially available crystalline amino acids. Thus, the pigs' requirement for these indispensable amino acids can be fulfilled by directly supplying these crystalline amino acids. However, when the need for the first 2 to 3 limiting amino acids is fulfilled, most of the other indispensable amino acids may become limiting. Thus, after fulfilling the requirement for Lys and Thr by the inclusion of the crystalline amino acids, Ile, Met, His, Leu, Val and Phe become the next limiting amino acids in dry stored barley. In this case, the supplementation of crystalline Met and Val to dry stored barley would become meaningless because they are not be used for protein synthesis if the requirement for Ile is not fulfilled. in barley diet Thus, the digestibility of non-commercially available indispensable amino acids (Ile, Leu, His, Phe and Arg) becomes the limiting factors to improve N and amino acid utilisation of barley because these amino acids can only be supplied by protein feedstuffs, e.g. SBM and rapeseed meal. Interestingly, adding the enzyme combination to HMA stored barley enhanced the AID of not only Lys and Met but also of Arg and tended to increase the AID of Ile, Leu and Phe (Manuscript III). HMA stored barley with the enzyme combination also contained a significant higher amount of digestible Lys, Met+Cys, Ile and Phe and a numerically higher digestible amount of other indispensable amino acids except Thr and Arg (Manuscript III).

Ammonia emission is of environment concern and mainly related to the intensive pig production. Ammonia emission is primarily associated with the excretion of N via urine caused by the imbalance in amino acid supply in pig diets. The principle of protein synthesis in animals is “all or nothing” that requires all amino acids to be available in the proper amounts or none can be used (Kang, 2013; Tanksley and Knabe, 1993). In the present study, although the enzyme combination enhanced the AID of Lys, the first limiting amino acid of barley, the amino acid profile of barley was still imbalanced and limited in Lys content. As long as the requirement of Lys is not fulfilled, the increase in AID and digestible amounts of the other indispensable amino acids and especially of the
dispensable amino acids by HMA storage and the enzyme combination lead to a higher amount of unretained amino acids. In fact, the increased AID of N (12% points) and total amino acids (5% points) of barley by the enzyme combination was mainly due to the significant enhancement in the AID of dispensable amino acids (8%). Consequently, the tendency towards a higher urinary N excretion in pigs fed the barley diets with the enzyme combination may be due to the increased absorption of the dispensable amino acids and to some extent also the dispensable amino acids, which are deaminated and excreted via urea. Additionally, the increase in urinary N excretion may be related to the degradation of NSP in barley caused by the enzyme combination. The decrease in fermentable NSP like β-glucan also reduces the secretion of urea from the blood due to a lowered microbial growth resulting in a lower microbial protein synthesis and a higher reabsorption of ammonia from the colon which shift the N excretion from faeces to urine (Canh et al., 1997; Zervas and Zijlstra, 2002). Humer et al. (2014) also observed a higher ratio of urinary N to faeces N in pigs fed HMA stored maize. The breakdown of urea in urine is the main source of ammonia emission from pig manure, whereas only a small part originates from the degradation of protein in faeces (Aarnink et al., 1993; Aarnink and Verstegen, 2007). Therefore, the shift in N excretion from faeces to urine, all things be equal, contributes to a higher ammonia emission. However, the current barley diets contained solely barley as the N and amino acid source. Thus, with a proper use of crystalline amino acids and complementary CP, the enhancement in AID of indispensable amino acids by HMA storage with the enzyme combination may lead to a reduction in ammonia emission.

3. Relationship between nutrient solubility and digestibility

The 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 in the small intestine. Phytate P is the main source of insoluble P that influence the ATTD of P of cereals; thus, P solubility – indicating the release of P from phytate – has been used to predict the ATTD of P of cereals in pigs (Columbus et al., 2010; Niven et al., 2007). HMA storage and enzyme combination enhanced both the P solubility and the ATTD of P of barley in the current study (Manuscript II). As the P solubility increased by averaged 53% and 11% points, respectively in response to HMA storage and the enzyme combination (Manuscript II), the ATTD of P was also increased by 15% and 13% points, respectively in pigs (Manuscript II). In agreement with current study, Columbus et al.
(2010) observed that phytase addition increased the P solubility from 0.20 to 0.26-0.28 which is much lower compared with the current study (from 0.23 to 0.35-0.87) and may explain the insignificant increase in ATTD of P (2-7% points) in their study. These findings confirmed that an increase in P solubility (to a certain extent) is an indicator for the enhancement in ATTD of P in pigs.

N solubility is considered to be a sensitive marker of the mobilisation of protein during HMA storage (Åman et al., 1990a). The in vitro method predicting the AID of protein and amino acids in feedstuffs for pigs by Qiao et al. (2004a) also claimed that the soluble protein is more digestible compared with insoluble protein in vivo. The increase in protein solubility of barley before feeding is therefore expected to result in a higher amount of digestible protein and amino acids in diets. Soaking of the same barley cultivar (Zephyr) as used in the current study increased the protein solubility by 15-22% points and thereby enhanced the ATTD of N by 3% points (Christensen, 2013b). In the current study, the enhancement in N and protein solubility by HMA storage did not result in a corresponding increase in the ATTD and AID of N but resulted in a higher AID of Met and Arg (5% points) (Manuscript III). This may be partly explained by the smaller increase in protein solubility (4-6% points) in HMA stored barley compared with that in study of Christensen (2013b). This underlines the use protein solubility as an indicator to predict the N and amino acids digestibility in pigs. Moreover, the enzyme combination enhanced the AID of N and amino acids in dry stored barley to the same extent as in HMA stored barley (Manuscript III). Thus, the increased N and protein solubility in barley by HMA storage and the enzyme combination before feeding seems to have a similar small impact on the AID of N. In fact, the enzyme combination may have solubilised protein and amino acids of dry stored barley in the proximal part of the digestive tract to the same extent as that of HMA stored barley.

Overall, the solubility of P and protein of cereals can be used as indicators to predict the digestibility of P and N of cereals in pigs.
Chapter VII. **Conclusion and Perspective**

1. **Conclusion**

   The PhD study aims to explore the effect of high moisture airtight (HMA) storage with a combination of phytase, xylanase, β-glucanase and protease on enhancing the phytate degradation, the P and N solubility of barley and triticale and the digestibility of P, N and amino acids of barley in pigs. The following conclusions can be drawn from the study:

   i. It is necessary to process grain (rolling) before storage and adjust grain moisture content to minimum 26% and preferred to above 30% to achieve the increase in phytate degradation and the solubility of P and N in HMA stored barley and triticale compared with dry stored barley and triticale.

   ii. 35% moisture seems to be the optimum moisture level for HMA storage of rolled barley to enhance the phytate degradation and the solubility of P, N and protein compared with dry storage and at the same time to prevent the loss of energy and N via ammonia by microbial fermentation.

   iii. The inclusion of the enzyme combination during HMA storage for 49 days significantly increased the phytate degradation and the solubility of P and N of rolled barley (29%, 35% and 40% moisture) and rolled triticale (29% moisture) to a greater extent compared with no enzyme addition.

   • **Take-home message 1:** HMA storage with the enzyme combination enhanced the phytate degradation and the solubility of P and N of barley and triticale.

   iv. HMA storage of rolled barley at 35% moisture with enzyme combination resulted in the highest enhancement in ATTD of P in barley diet (up to 70%) compared with dry storage.

   v. When barley was fed together with SBM, HMA storage of barley at 35% with the enzyme combination for 49 days enhanced the ATTD of P to a smaller extent (36%) compared with feeding solely barley.

   vi. HMA storage of barley with the enzyme combination shifted the P excretion from faeces to urine because of the low daily Ca intake (<1g/d) in barley and barley-SBM diets.

   • **Take-home message 2:** High moisture airtight storage with enzyme combination enhanced the apparent total tract digestibility of P of barley in growing pigs.
vii. HMA storage of rolled barley at 35% moisture with enzyme combination for 49 days enhanced the AID of N (16%), the AID of the indispensable amino acids (Lys, Met and Arg, 10-15%) and the amount of digestible indispensable amino acids (Lys, Met+Cys, Ile and Phe, 7-17%) compared with dry storage in pigs fed barley diets.

viii. Storage of barley (HMA storage vs. dry storage) and enzyme combination (without vs. with) did not affect the overall ATTD and AID of N and the AID of amino acids in pigs fed barley together with SBM.

ix. HMA storage of rolled barley at 35% moisture with enzyme combination for 49 days had no effect on reduce the excretion of N via manure compared with dry storage in pigs fed barley alone or in combination with SBM.

➢ **Take-home message 3**: HMA storage with the enzyme combination enhanced the apparent ileal digestibility of N and amino acids of barley in pigs

x. The P and protein solubility of barley can be used as an indicator to predict the ATTD of P and the AID of N of barley, respectively in pigs. However, it is not possible to judge between the P and protein solubility which one is a more accurate indicator.

➢ **Take-home message 4**: The solubility of P and N are indicators to predict the digestibility of P and N in pigs

2. **Perspective**

This PhD study is the first study that explored the effects of HMA storage with the enzyme combination and provided addition knowledge on HMA storage in the relationship with other factors (grain processing, grain moisture, storage time and cereal type). The enhancement in the P and N solubility and digestibility by HMA storage of barley with the enzyme combination resulted in a reduction in the faecal P excretion, but no effect on the total excretion of P and N to manure in pigs. This reveals the possibility to maximise the effect of HMA storage and the enzyme combination towards a higher enhancement in the digestibility of P, N and amino acids and at the same time a lower excretion in P and N. A reduction in P and N excretion via manure will alleviate the environmental impacts of intensive pig production, e.g. decreasing ammonia emission and P and N leaching.

The current study proved that barley and triticale responded differently to HMA storage and the added microbial enzymes. Thus, it would be of interest to investigate the effect of HMA storage in various cereals and to evaluate the correlation between the
nutrient digestibility and the grain moisture content in order to determine the optimum storage conditions for each cereal. Based on this, it will be possible to select cereal cultivars suitable for HMA storage.

Moreover, the current study concluded that the enhancement in the phytate degradation and the nutrient solubility of cereals by HMA storage is mainly due to the endogenous enzymes in cereals and to a limited extent by the microbial fermentation. To confirm the extent of microbial fermentation during HMA storage, other parameters should be measured in future studies, e.g. organic acid content (lactic acid, acetic acid and butyric acid), bacteria count and ammonia concentration.

The inclusion of phytase, xylanase, β-glucanase and protease in combination during HMA storage enhanced the nutrient solubility and digestibility of cereal. However, it was impossible to identify which enzyme in the combination play the vital role on the enhancement. Thus, further research should be done to identify the role of each enzyme and their interaction on enhancing nutrient availability of HMA stored cereals. Consequently, it will be possible to select the most effective enzymes, the dose of enzyme supplementation and the optimum storage conditions to maximise the enhancement in nutrient digestibility of cereals in pigs.
References


Bender, D. A. 2007. Introduction to nutrition and metabolism. 4th ed. CRC Press, Florida, USA.


Christensen, J. B. 2013a. Exploiting the enzyme potential of cereals in liquid feed for pigs: possibilities and limitations, Aarhus University, Tjele.


barley during different high moisture storage conditions. Animal Feed Science and Technology 64: 257-272.


