In vivo and *in vitro* studies of degradation of inositol phosphates in the digestive tract of broiler chickens

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AA(s)	Amino acid(s)
avP	Available phosphorus
Ca	Calcium
Cl	Chlorine
DM	Dry matter
FCR	Feed conversion ratio
Fe	Iron
FTU	Phytase unit
G:F	Gain-to-feed ratio
HCl	Hydrochloric acid
HSC	Highly soluble calcified seaweed
InsP(s)	Inositol phosphate(s)
$InsP_1$	*Myo*-inositol monophosphate
$InsP_2$	*Myo*-inositol bisphosphate
$InsP_3$	*Myo*-inositol trisphosphate
$InsP_4$	*Myo*-inositol tetrakisphosphate
$InsP_5$	*Myo*-inositol pentakisphosphate
$InsP_6$	*Myo*-inositol hexakisphosphate
K	Potassium
LOQ	Limit of quantification
MCP	Monocalcium phosphate
Mg	Magnesium
MI	*Myo*-inositol
Mn	Manganese
Na	Sodium
n.d.	Not detectable
NPP	Non-phytate-phosphorus
P	Phosphorus
P_i	Inorganic phosphorus

PP	Phytate- or $InsP_6$-phosphorus
Ti	Titanium
WPSA	World's Poultry Science Association
Zn	Zinc

1 EXTENDED INTRODUCTION

Phosphorus (P) is an essential element for the growth of animals. About 80% of total body P is deposited in the bones. The remaining 20% can be found in soft tissues and fluids. Phosphorus is a constituent of deoxy- and ribonucleic acids (DNA and RNA), and bound as adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP), P is involved in the energy utilization of cells. Bound as phospholipid, P participates in the integrity and fluidity of cell membranes (Suttle, 2010). To ensure optimal growth and well-being of animals, P must be adequately supplied with the feed. Usually, the P supplemented to poultry diets is derived from phosphate rock. Phosphate rock, however, is a non-renewable resource that could be depleted within the next 50-100 years (Cordell *et al*., 2009). A 'peak phosphorus' is predicted to occur around 2033. Peak phosphorus is defined as the maximum of production after which the quality and accessibility of rock phosphates will decrease, making it uneconomical to mine and process, and this will consequently lead to a decreasing supply of P. Simultaneously, however, the demand for rock phosphate will rise (Cordell *et al*., 2009) as the world population and the demand for meat increases. The world population is estimated to increase by 25% between 2015 and 2050, while the meat consumption per capita is estimated to increase by 13% in developed and 38% in developing countries during this time span. Consequently, the total meat consumption is estimated to increase by 53% between 2015 and 2050 (Thornton, 2010). Chicken meat provides a high-quality protein source, and these animals have the highest feed efficiency among all farm animals designated for meat production, illustrating the future relevance of chickens. A further issue is that only five countries control over 90% of global P reserves, with Morocco controlling about 77%. The other four countries are China, South Africa, Jordan and the USA (Neset and Cordell, 2012). Europe has hardly any P reserves and the ban on meat-and-bone meals in the European Union in the year 2000 has accentuated the problem of insufficient P supply. As seen in FIGURE 1, a substantial amount of the mined phosphate rock is needed for domestic animals, either as fertilizers for feed plants or by direct use as feed P. On the other hand, the loss of P from manure is quite high. Almost half of the P in manure is lost in soils, landfills, and waters. This in turn might lead to the eutrophication of lakes (Rodehutscord, 2009). The identification of sustainable future pathways and the contribution of research in different fields are now more important than ever. This entails an increase in efficiency

(Thornton, 2010), and in particular the optimization of the P supply of livestock (Rodehutscord, 2008). The P requirement of poultry could—in most cases—be met with the P contained in the plant seeds that are the main component of poultry rations, but only under the assumption of a complete availability of the plant P.

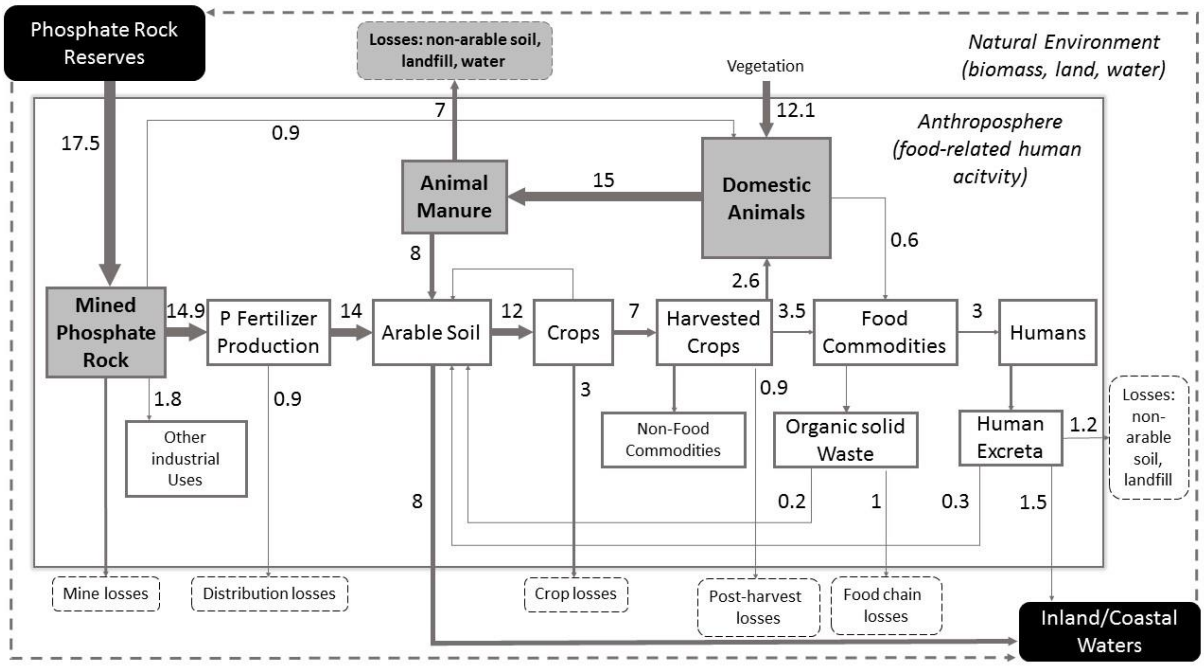

FIGURE 1. Key P flows through the global food production and consumption system, indicating P usage, losses and recovery at each key stage of the process. Units are in million tons per year. Animal-related stages are highlighted in grey color. Graphic adapted from Cordell *et al*. (2009).

However, the P in plant seeds is mainly bound as *myo*-inositol (1,2,3,4,5,6)-hexakis (dihydrogen phosphate) (phytic acid or $InsP_6$), where $InsP_6$-P (PP) can make up 70% of total P, with great variation between grain types (Eeckhout and de Paepe, 1994; Rodehutscord *et al*., 2016). Phytic acid is unstable in its free form, and thus mainly occurs in its stable salt form, called phytate (Konietzny and Greiner, 2003). Therefore, the terms phytate and $InsP_6$ will be used interchangeably in this thesis. As salt, phytate can form complexes with cations like calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), and zinc (Zn) (Humer *et al*., 2015). Bound as phytate, P is stored in the seeds until its enzymatically catalyzed release during the germination process (Loewus and Loewus, 1983). Phosphorus can be cleaved from $InsP_6$ by phytate-degrading enzymes. In contrast to ruminants, which benefit from microorganisms able to produce phytate-degrading enzymes in their rumen (Haese, 2017), the $InsP_6$-degrading enzyme equipment of non-ruminants is limited. Thus, PP is only

partially available for non-ruminants. As a result, poultry diets are mostly supplemented with mineral P to meet their requirements. The proportion of PP that cannot be cleaved from the phytate molecule and used by the animal, is excreted. This in turn leads to the aforementioned accumulation of P in soils and consequently to the eutrophication of lakes. To make matters worse, the supplemented P reduces the proportion of P that could potentially be cleaved from the phytate molecule (Zeller *et al*., 2015c), which leads to an even greater proportion of unutilized—thus excreted—PP. $InsP_6$ is not only critical in terms of P provision for non-ruminants, it can also form complexes with other nutrients and consequently make them less available for animals. Due to its 12 replaceable reactive acidic sites and its negative charges at pH values prevailing in the small intestine, $InsP_6$ can bind bi- and trivalent cations in stable complexes (Angel *et al*., 2002). The affinity of $InsP_6$ to complex cations and the strength of these complexes are found to be in the following order: $Cu^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}$ (Singh, 2008; Vohra *et al*., 1965). Although $InsP_6$ forms the weakest complexes with Ca among all other multivalent cations, these complexes have the highest relevance for poultry as the concentration of Ca in the diets is much higher than those of the other cations. The complexation of $InsP_6$ and Ca and thus the solubility of both are highly dependent on the pH values and the Ca:$InsP_6$ molar ratios as shown by Grynspan and Cheryan (1983). Both were highly soluble below pH 4. As reviewed by Selle *et al*. (2009a), it is possible that Ca forms complexes with both $InsP_6$ and phosphate as visualized with the following equation:

$$Ca\text{-}InsP_6\ complexes \leftarrow InsP_6 + Ca + PO_4^{3-} \rightarrow Ca_3(PO_4)_2$$

Whether Ca-$InsP_6$ or Ca-phosphate is preferably formed depends on the molar ratios of $InsP_6$ and phosphate. However, Ca tends to prefer complexation of $InsP_6$ over phosphate. Apart from cations, $InsP_6$ can also form complexes with other nutrients like starch, proteins and amino acids (AAs), inhibit digestive enzymes and reduce fat digestibility. The possible mechanisms leading to $InsP_6$-protein complexes and how $InsP_6$ and phytase can alter AA digestibility in broiler chickens are discussed in the GENERAL DISCUSSION chapter.

In literature, studies differ in their description of the binding form or availability of P. To avoid confusion, the terminology that appears in the GENERAL DISCUSSION chapter and that is described by WPSA (2013) is explained here briefly.

- Total P is the whole P contained in the feed irrespective of its binding form, which can be measured by chemical analysis.
- Phytate-P (PP) is the proportion of total P that is bound to $InsP_6$ which can be measured by chemical analysis.
- Non-phytate-P (NPP) is the proportion of total P that is not bound to $InsP_6$
  $\rightarrow$ PP + NPP = total P.
- Available P (avP) is the proportion of total P that can be utilized by the animal at low P supply level. It consists of inorganic P (P_i), and organic P, mostly freed from the $InsP_6$ molecule. It cannot be analyzed chemically and has to be determined experimentally.

$InsP_6$-degrading enzymes, called phytases (*myo*-inositol hexakisphosphate phosphohydrolases), cleave P from the phytate molecule. During the dephosphorylation of phytate, lower inositol phosphate (InsP) esters are formed with different levels of phosphorylation ($InsP_5 \rightarrow InsP_4 \rightarrow InsP_3 \rightarrow InsP_2 \rightarrow InsP_1$). Depending on the phytase and thus the preferential site of P cleavage, different isomers are created. For example, in the notation of the isomer Ins(1,2,5,6)P_4, the figures in the brackets indicate the position of the four P groups on the inositol ring in D-numbering. This, in turn, implies that two P groups were cleaved from Positions 3 and 4. As is apparent from FIGURE 2, the hydroxyl group on Position 2 is axial whereas all other hydroxyl groups are equatorial. The inositol molecule has a plane of symmetry through Positions 2 and 5. This means that Ins(1,2,5,6)P_4, for example, can be converted to Ins(2,3,4,5)P_4 by reflection in the plane of symmetry. Thus, they are enantiomers by definition. As enantiomers co-elute in high pressure ion chromatography (HPIC), they cannot be separated and are therefore quantified together. Although the D-numbering is commonly used in studies, it may happen that InsPs are presented in L-numbering. D-Ins(1,2,5,6)P_4, following the numbers in the black circles in FIGURE 2, can therefore also be labelled as L-(2,3,4,5)P_4, following the numbers in the grey circles in FIGURE 2, but the two are the identical molecule. Likewise, D-(2,3,4,5)P_4 can be labelled as L-(1,2,5,6)P_4, also being an identical molecule (Irvine and Schell, 2001).

Phytases are classified by their catalytic properties into purple acid phytases, β-propeller phytases, cysteine phytases or histidine acid phosphatases, whereby the latter provides the majority of the phytases known today. Further, they can be classified based on their pH optimum into acid or alkaline phytases. The classification most commonly applied is based on

the C-atom of the inositol-ring at which the phytase starts the dephosphorylation. Three classes are recognized by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB) to date: 3-phytases (E.C. 3.1.3.8), which cleave the first P residue from position D-3 (L-1), 5-phytases (E.C. 3.1.3.72), cleaving the first P residue from position D-5 (L-5), and 6-phytases (E.C. 3.1.3.26), which cleave the first P residue from position L-6 (D-4) (Greiner and Konietzny, 2010). Commonly, 3-phytases were recognized to be of microbial origin and 6-phytases of plant origin (Greiner and Konietzny, 2010). In recent years, however, it has been shown that this does not hold for all cases.

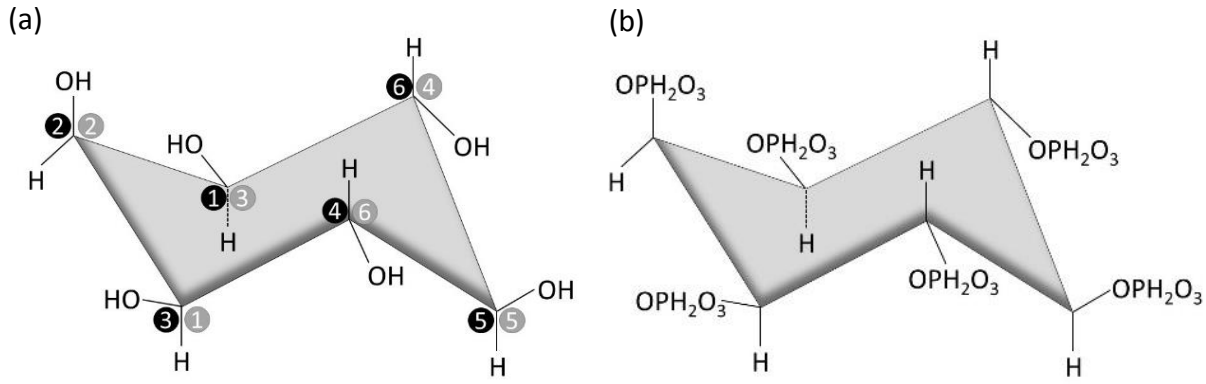

FIGURE 2. *Myo*-inositol (a) and *myo*-inositol (1,2,3,4,5,6)-hexakis (dihydrogen phosphate) ($InsP_6$) (b) in the most stable conformation *in vivo*: the "chair" structure. Numbers in black circles represent the D-numbering (anticlockwise). Numbers in grey circles represent the L-numbering (clockwise). (Adapted from Irvine and Schell (2001)).

Activities of 3-phytase have been found in plant seeds (Greiner, 2002) and 6-phytase activities in microorganisms, for example *Escherichia coli* (Greiner *et al*., 1993). It must also be noted that the aforementioned 6-phytases of plant origin initiate the phosphorylation at the D-4 (L-6) position. According to the general rule to label the phytate molecule in the D-numbering, plant phytases are 4-phytases, strictly speaking. The mentioned microbial 6-phytases, however, are labeled according to the D-numbering and have to be strictly separated from the plant 4-phytases (Greiner and Konietzny, 2010). The most common phytase classes used in the nutrition of non-ruminants are 3- and 6-phytases. Activity of 5-phytases has only been detected in lily pollen (Barrientos *et al*., 1994; Mehta *et al*., 2006), *Selenomonas ruminantium* subsp. *lactilytica (Puhl et al*., 2008), and *Bifidobacterium pseudocatenulatum* (Haros *et al*., 2009). Greiner and Konietzny (2010) suggested that 2-phytases must be present in animal cells because partly dephosphorylated InsPs were found to be initially dephosphorylated at

position D-2. And although no 1-phytases are known yet, it is hypothesized that these might also be present in nature. Phytases are per definition $InsP_6$ hydrolases. Phytase activity is expressed as phytase units (FTU) whereby one FTU is commonly defined as the activity of the phytase to liberate 1 µmol P_i from sodium (Na) phytate (c=0.0051 mol/L) per minute at 37 °C and pH 5.5 (Qvirist *et al*., 2015). However, phytases can also dephosphorylate lower InsP esters. It is suggested that phytases first degrade all available $InsP_6$ molecules to $InsP_5$ esters before they start dephosphorylating the $InsP_5$ esters to $InsP_4$ esters (Dersjant-Li *et al*., 2015; Yu *et al*., 2012). However, there is some evidence that at conditions that decrease the solubility and thus the availability of $InsP_6$—for example high Ca concentrations—phytases can change their preference toward degrading lower InsP esters before $InsP_6$ is entirely degraded to $InsP_5$ (Pontoppidan *et al*., 2007).

Phytases are widely distributed in plants, animals, and microorganisms. Intrinsic plant phytases are present in grains, seeds, and pollen of higher plants. They are of use in the process of germination, when the plant needs P, minerals and *myo*-inositol (MI) for growth. In grain seeds, phytase activity varies between very low (< 200 U/kg dry matter (DM)) in oats, corn, and soybeans to very high (> 1,500 U/kg DM) in rye, triticale, and wheat (Eeckhout and de Paepe, 1994; Rodehutscord *et al*., 2016) with great variations between genotypes within one grain species (Rodehutscord *et al*., 2016). Plant phytases are known to be susceptible to heat (during the pelleting process), low pH (in the stomachs of birds) and digestive proteins (Phillippy, 1999; Scholey *et al*., 2017). In the studies by Zeller *et al*. (2015a, 2016), for example, the $InsP_6$ disappearance in the crop was higher in broiler chickens that were fed a diet based on wheat than in broiler chickens that were fed a diet based on wheat with inactivated intrinsic phytase. However, the $InsP_6$ disappearance up to the terminal ileum did not differ between the treatments.

The presence of endogenous phytases, which are able to degrade $InsP_6$ in the digestive tract of animals, is well-known. The potential of broiler chickens to degrade up to 89% $InsP_6$ up to the terminal ileum from a diet with negligible intrinsic phytase activity under P-deficiency has been shown by Zeller *et al*. (2015b), Applegate *et al*. (2003), Shastak *et al*. (2014), Leytem *et al*. (2008), and Tamim *et al*. (2004). There are two possible sources of endogenous phytases. The first potential source of endogenous phytases is the epithelial cells of the digestive tract. Phytase activities in epithelial cells of broiler chickens were observed by Maenz and Classen

(1998) and Huber *et al*. (2015), among others, by incubating brush border membrane vesicles of the small intestine with $InsP_6$ and subsequently measuring the released P_i. The activity of phytase in the mucosa of the small intestine was shown to decrease between the duodenum and the ileum and to be pH-dependent, with decreasing activity above pH 6.6 (Abudabos, 2012; Maenz and Classen, 1998). The phytase activity in the digestive tract increases with the age of the birds (Marounek *et al*., 2008; Nelson, 1967; Singh, 2008).

Another potential source of endogenous phytases is the microorganisms residing in the digestive tract. Kerr *et al*. (2000) reported much higher $InsP_6$ concentrations in the digesta of the ceca of gnotobiotic broiler chickens than in conventional chickens. They concluded that the microorganisms make a high contribution to the $InsP_6$ degradation in the hindgut. Moreover, many microorganisms have already been identified as phytase producers of which several inhabit segments of the digestive tract of broiler chickens (Borda-Molina *et al*., 2016; Greiner and Konietzny, 2010; Witzig *et al*., 2015). For example, phytase and phosphatase activities were found in different *Lactobacillus* strains (Haros *et al*., 2008) and Lactobacillaceae was found to be the most abundant family in the crop, jejunum, and ileum of broiler chickens (Witzig *et al*., 2015). However, until now, it has not been possible to separate and quantify the contribution of these two sources of endogenous phytases.

Exogenous microbial phytase products, derived from bacteria and fungi, have been commercially available since 1991. It started with the introduction of an *Aspergillus niger* phytase; soon, more phytases of different origins entered the market. Nowadays, they are widely used in poultry and swine nutrition (Dersjant-Li *et al*., 2015). As the production or activity of phytases is generally very low in wild strains of bacteria, their phytase genes are isolated, cloned and overexpressed in host organisms (Jain *et al*., 2016). As properties of phytases from the same donor organism but different production organism may vary, it is important to mention not only the donor organism (for example *E. coli* or *A. niger*) but also the production organism (Menezes-Blackburn *et al*., 2015; Tran *et al*., 2011). Microbial phytases can vary in their pH optimum and susceptibility to digestive enzymes like pepsin and pancreatin; for example, fungal phytases seem to be more susceptible to pepsin than bacterial phytases (Menezes-Blackburn *et al*., 2015).

$InsP_6$ disappearance and the appearance of lower InsP esters and their isomers in different segments of the digestive tract were shown to be dependent on the P and Ca content of the

diets (Shastak *et al*., 2014; Zeller *et al*., 2015c). Supplementation of P and Ca reduced the degradation of InsPs in the studies by Shastak *et al*. (2014) and Zeller *et al*. (2015c); this was observable with and without a supplemented microbial phytase and in all segments of the digestive tract. Thus, supplementation of P and Ca could have had diminishing effects on phytases from all the sources described here. The reasons for these outcomes might be an inhibition of phytases by the end product P (Greiner *et al*., 1993; Zeller *et al*., 2015c) or the decrease in the solubility of $InsP_6$ due to supplemented Ca (Tamim *et al*., 2004; Zeller *et al*., 2015c). However, as P and Ca mostly are supplemented in combination, a clear discrimination between the effects derived from P or from Ca has not been made so far. The effects of dietary P and Ca on $InsP_6$ disappearance and the appearance of lower InsP esters in the digestive tract of broiler chickens are discussed further in the GENERAL DISCUSSION chapter.

Due to the lower affinity of $InsP_{1-4}$ for proteins (Yu *et al*., 2012) and metal ions (Persson *et al*., 1998), it would be of benefit if the supplemented phytase were to degrade the phytate in the anterior digestive tract segments to lower InsPs. Moreover, lower InsPs can be further dephosphorylated by endogenous phosphatases secreted from the epithelial cells in the small intestine (Kemme *et al*., 2006). The activity of phytases, the abundance or activity of microbial groups, and the complexation of Ca with $InsP_6$ are known to be pH-dependent. Thus, a microbial phytase with a pH optimum range at the pH value prevailing in the crop and stomachs would be beneficial for achieving an early dephosphorylation of $InsP_6$ to lower InsP esters. An increase in pH due to a higher Ca inclusion level in the diet was observed in the crop (Shafey and McDonald, 1991), the gizzard (Amerah *et al*., 2014; Morgan *et al*., 2014b; Walk *et al*., 2012c), and the ileum (Shafey and McDonald, 1991; Walk *et al*., 2012c). A pH increase due to high phytase inclusion levels was reported for the crop (Amerah *et al*., 2014; Ptak *et al*., 2015), the gizzard (Amerah *et al*., 2014; Walk *et al*., 2012c), and the small intestine (Amerah *et al*., 2014; Walk *et al*., 2012c). The pH-increasing effect of high phytase concentrations can possibly be explained by a reduced hydrochloric acid (HCl) secretion in the gizzard due to degraded $InsP_6$ in the anterior segments (Amerah *et al*., 2014). Lawlor *et al*. (2005) investigated the acid-binding and buffering capacity in the stomach of pigs for a variety of feedstuff. The result in regard to feedstuff commonly used in poultry nutrition is a high acid-binding capacity and buffering capacity for soybean, sunflower, and rapeseed meal, whereas the acid-binding capacity and buffering capacity of wheat and corn is at least four times lower.

This could also have effects on the pH in crop and stomachs of poultry and thus phytase activity and InsP degradation in the anterior segments of the digestive tract depending on the composition of the diet. However, these results are not consistent throughout studies and indicate that the change in intestinal pH is based on an interplay of several factors.

Due to the addition of phytase, not only can the complex formation of $InsP_6$ be diminished, but also the formed lower InsP esters and their isomers are known to fulfill several functions inside the animal's body. The isomers Ins(1,4,5)P_3, Ins(1,2,6)P_3, and Ins(1,3,4,5)P_4 are considered to be involved in intracellular regulation processes, by releasing intracellular Ca for example (Luttrell, 1993; Shears *et al*., 2012). Irvine and Schell (2001) suggested that Ins(3,4,5,6)P_4 is involved in chlorine (Cl) secretion. Ins(1,3,4,5,6)P_5 is a constituent of avian erythrocytes, enhancing the affinity of erythrocytes for oxygen (Irvine and Schell, 2001). Further, InsPs phosphorylated at Positions 1, 2 and 3 are shown to inhibit Fe-catalyzed hydroxyl radical formation (Phillippy and Graf, 1997). Even several enteropathogens are able to utilize InsP esters for their survival and replication (Heyer *et al*., 2015). However, whether and to what extent $InsP_6$ and lower InsP esters are absorbed during the passage through the digestive tract is not clear. Sakamoto *et al*. (1993), based on their study with radiolabeled $InsP_6$ administered to rats, suggested that $InsP_6$ is absorbed rapidly, mainly in the anterior parts of the digestive tract, including the stomach, followed by distribution throughout the body. However, specific $InsP_6$ transporters have not yet been found in mammals and a diffusion is hardly possible due to its polarity (Wilson *et al*., 2015).

After the complete dephosphorylation of the $InsP_6$ molecule, six P residues and MI are potentially available for the animal. The supplementation of high phytase doses can lead to an increase in MI concentration in the gizzard (Beeson *et al*., 2017; Walk *et al*., 2014), in the ileum and excreta (Beeson *et al*., 2017), and in the blood plasma (Cowieson *et al*., 2015). These results suggest that MI released by phytase from $InsP_6$ is subsequently absorbed from the digestive tract. Indeed, the specific transport of MI from the intestine through the apical wall was found to take place via an Na-dependent SMIT2 transporter in rats with a high affinity for MI (Aouameur *et al*., 2007). The Na-dependence of MI transport has already been investigated by Caspary and Crane (1970), Lerner and Smagula (1979), and Scalera *et al*. (1991) by incubation of segments of hamster, broiler, and rat small intestines. They also found that sugars are able to inhibit MI transport without having the same transport system. It seems

that MI uptake is highly dependent on the individual bird, as the influx values ranged between 2 and 113 nmol/g intestine per 10 minutes in more than 100 birds without being affected by age in the study by Lerner and Smagula (1979). Once in the blood, MI can be transported and taken up by the brain, liver, kidney, and other tissues (Huber, 2016; Lee and Bedford, 2016).

Myo-inositol is known to have several functions in the body (Huber, 2016; Lee and Bedford, 2016). As summarized by Huber (2016), MI acts as precursor for $InsP_3$, which is a second messenger responsible for Ca^{2+} release and endocrine signaling, and for $InsP_5$, which is present in avian erythrocytes and functions as a modulator of hemoglobin oxygenation (Isaacks *et al*., 1989). Further, MI acts as an osmolyte in the brain, bone marrow, and liver. Bound as phosphatidylinositol phosphate, it is involved in cell migration, signal transduction, and trafficking (Huber, 2016). In the study by Huber *et al*. (2017), a positive relationship was found between the MI concentration in blood plasma and serotonin and dopamine in trunk blood. This suggests that MI can have effects on the behavior of birds. As MI has a variety of functions in the body, its supply does not only depend on the provision via feed intake; it can also be synthesized *de novo* from glucose. Latest research on MI in broiler chicken reveals MI as a potential factor for enhancing the performance and feed efficiency of the birds, concluding that the release of MI might be one of the beneficial effects of phytase apart from the release of P. A deeper insight into the studies investigating the effects of MI on the performance of broiler chickens is provided in the GENERAL DISCUSSION chapter.

Based on the overarching aim to optimize P utilization in farm animals as a contribution to the P crisis, and the background presented here, it is obvious that more research is needed in the field of phytate degradation. Apart from the release of P, which contributes to the P supply of the animal, there are many more benefits of the degradation of $InsP_6$. The formation of lower InsP esters and the release of MI and other nutrients like minerals and AAs during $InsP_6$ degradation can contribute to an optimal supply of nutrients and thus the growth of the animals. However, $InsP_6$ degradation is affected by several factors, which need to be investigated to achieve maximum benefits for the animal, the environment, and the poultry producer.

2 OVERVIEW AND AIMS OF THE INCLUDED STUDIES

The overarching aim of this thesis was to gain a deeper insight into the degradation of $InsP_6$ in broiler chickens, with a focus on the intermediate and end-products as influenced by the diet composition.

Aiming to reduce the number of animals used for *in vivo* experiments, an *in vitro* assay was established to evaluate feed supplements and possible affecting factors. In contrast to the standard phytase assay, the present assay simulates the conditions prevailing in the digestive tract of poultry, but without the interference of endogenous phytases (MANUSCRIPT 1). The *in vitro* assay enables us to test enzymes and other supplements in a feed matrix under standardized conditions. In this *in vitro* assay, the effects of P, Ca, and phytase supplementation on the InsP degradation in a broiler diet were investigated.

The next step was to investigate single or interactive effects of P, Ca and phytase *in vivo*. The experimental design arose from the outcomes of previous experiments by our working group, which reported diminishing effects of a combined P and Ca supplementation on InsP degradation and prececal P digestibility (Shastak *et al*., 2014; Zeller *et al*., 2015c). In this experiment, the effects of a supplementation of P, Ca, and phytase alone or in combination were studied on the following traits in broiler chickens: InsP degradation and appearance of lower InsP isomers in different segments of the digestive tract, the concentration of MI in different segments of the digestive tract and the blood, and the prececal AA digestibility (MANUSCRIPT 2). Due to the advancement of analytical methods by our working group, the detection of all InsP esters ($InsP_{1-6}$) with respective isomers in the digesta, and of MI in digesta and blood has been made possible. Therefore, a complete picture was drawn of the degradation steps between $InsP_6$ and MI in the ileum.

Another *in vivo* trial was carried out based on the outcome of the latter trial, where significant effects of P, Ca, and phytase on the MI concentration in the digestive tract and blood were found. The effect of the supplementation of graded levels of phytase and MI, and thus the potential effects of released or free MI on performance, InsP degradation, and prececal P, Ca, and AA digestibility were studied (MANUSCRIPT 3).

3 GENERAL DISCUSSION

The aim of the present work was to gain a deeper insight into the degradation of InsPs in the digestive tract of broiler chickens and investigate the possible diminishing factors. Previous experiments by the working group of Prof. Dr. Markus Rodehutscord formed the basis for the research on degradation products of $InsP_6$ in the broiler's digestive tract. They were the first to investigate the degradation steps of $InsP_6$ with the differentiation of InsP isomers in different digestive tract segments, depending on feed matrix, composition, and enzymes. Based on the outcomes of these experiments, the question arose as to whether the diminishing effect of a monocalcium phosphate (MCP) supplementation on InsP degradation can be attributed to the supplemented P or Ca or a combination of the two. This issue should be addressed with two experiments described in this thesis. In the first experiment, the effect of supplemented P or Ca or both on InsP degradation was investigated *in vitro*. The *in vitro* assay simulating the digestive tract of poultry had to first be established. The advantages and limitations faced when working with such an *in vitro* assay are discussed herein. Following this, an *in vivo* experiment was carried out to investigate the single or interactive effects of P, Ca, and phytase supplementation on InsP degradation and the prececal AA digestibility in broiler chickens. For the first time, thanks to the advancement of methods in our laboratory, it was possible to analyze $InsP_{1-2}$ isomers and MI as an end product after the complete dephosphorylation of phytate. The effects of dietary Ca, P, and microbial phytase on MI concentration in different segments of the digestive tract and the blood are therefore discussed. *Myo*-inositol has gained more and more attention in recent years as it is believed to have the potential to increase the performance and feed efficiency of broilers. Based on the outcomes of the previous experiment and the advanced analytical methods, it was deemed logical to carry out an experiment with the supplementation of MI or graded levels of phytase. Therefore, the effects of MI on the bird, either added in its free form or released from phytate, are discussed.

3.1 Methodological considerations

There are several factors in an experiment that can influence the outcome and comparability of experiments, some of which are addressed herein. Factors related to the *in vitro* assay are discussed separately. The experiments described by Zeller (2015) were carried out according

to the same protocol that was applied in the present experiments. Thus, the methodical considerations relating to *in vivo* experiments investigating InsP degradation are already discussed in her doctoral thesis. For a better understanding of the present outcomes, however, the mentioned points are summarized here briefly. Further, as own results are compared to data in literature, some thoughts are expressed on the standardization and comparability of globally conducted digestibility experiments.

The contribution of the crop to $InsP_6$ degradation is still largely unknown. In birds, which are offered diets for *ad libitum* consumption, the ingested feed can bypass the crop. Usually, the crop serves as a storage organ that is utilized when feed is offered restrictedly (Classen *et al*., 2016). Birds in the present experiments had free access to feed; thus, there was no need to use the crop as a storage organ. However, they were kept on fast for one hour before sacrifice with one hour followed with access to feed. This means that—taken the retention times of the respective digestive tract segments into account—the digesta in the small intestine derived from before fasting and the digesta in the crop from after fasting. Thus, $InsP_6$ concentrations measured in the crop of these animals may not completely represent $InsP_6$ concentrations during the *ad libitum* feed intake.

$InsP_6$ disappearance was not calculated for the proventriculus and gizzard. Doing this would mostly lead to a higher value of $InsP_6$ disappearance than in the duodenum, jejunum, or ileum. The reason might be the inhomogeneous distribution of particles with different sizes in the stomachs and subsequent different flows of feed, InsP molecules, and the indigestible marker. Thus, the measured concentrations of nutrients are not well-represented by the marker (Perryman *et al*., 2017; Wilson *et al*., 2015). Vergara *et al*. (1989)—in an experiment comparing three different kind of markers (soluble or insoluble) with differing particle size—found that the particle size or type of the marker influences the disappearance of the marker not from the crop but from the stomach. This supports the suggestion that $InsP_6$ disappearance should not be calculated for the stomachs.

Another important point to consider in such experiments is the reverse peristalsis. Retrograde movements in the digestive tract can transport particles and microorganisms from the ceca up to the crop, as shown by Sacranie *et al*. (2007), thus affecting the concentrations of P, Ca, InsPs, MI, and the marker in all segments of the digestive tract.

The measured concentration of isomers of $InsP_{1-5}$ in the digesta is the result of different processes. It is derived from the feed and produced during the degradation of $InsP_{2-6}$ by different phytases and phosphatases; it may be absorbed or secreted into the lumen. Therefore, a calculation of the disappearance of lower InsP isomers with the help of the marker is not possible.

It is not known how the time between the taking of digesta samples and complete freezing affects the InsP degradation. The samples from all birds of one pen (up to 17 birds, depending on the experiment) are pooled, and although the people involved strive to work fast, several minutes can pass before the pooled sample is placed in the freezer. So far, it has not been investigated how the time between the opening of the birds and freezing the collected samples affects the activity of the intrinsic, endogenous, or added phytases and how this affects the InsP degradation in the digesta. It is therefore possible that the results of experiments may differ depending on the time needed by the sample to freeze to the core, making the present phytases inactive.

Standardization of experiments

In digestibility experiments, or more generally in scientific works, there is an attempt to standardize as many procedural steps as possible. The aim is to get results that are replicable and comparable with other experiments. To globally standardize experiments, the Working Group No 2 (Nutrition) of the European Federation of Branches of World's Poultry Science Association (WPSA) published a paper in which they proposed a framework for P-digestibility experiments (WPSA, 2013). This framework includes details about the experiment animals, general procedures, experimental diets, and calculations. However, after the recent publication of an international P-digestibility ring test (Rodehutscord *et al*., 2017), it is obvious that the results of prececal P digestibility and $InsP_6$ disappearance may vary enormously between stations even if the participants follow the WPSA protocol. This indicates that relevant factors are not considered in the protocol.

A similar collaborative study was carried out to investigate the prececal AA digestibility in broiler chickens at up to five different stations, following a protocol involving the sex and age of broilers, length and start of the experiment, sacrifice method, and collection and handling of samples (Ravindran *et al*., 2017). In the first run, they performed the experiments with each

station following its own protocol. Large differences were observed between the outcomes. However—in contrast to the P ring test—they observed a complete loss of variation between the results of the different research stations after application of the aforementioned protocol. Thus, it seems that the prececal digestibility of AAs is less susceptible to outer factors or can be better standardized than prececal P digestibility or $InsP_6$ disappearance.

The authors of the P-digestibility ring test provided a range of possible explanations, including the treatment of the birds during the starter phase or the influence of the microbiota. They concluded that it is now necessary to investigate the factors contributing to the observed high variation with subsequent adjustment of the present protocol for P-digestibility experiments.

The outcomes of these two studies have to be kept in mind when comparing P-digestibility or $InsP_6$-disappearance results of different studies. This also applies to AA-digestibility experiments. As the proposed protocol for AA-digestibility experiments was published in 2017, AA-digestibility studies carried out before that date should be compared with caution.

3.2 *In vitro* assays

When doing research with animals, everyone is supposed to follow the basic principle of the three Rs proposed by Russell and Burch (1959), defined as follows:

- *Replacement*—use alternatives to living animals
- *Reduction*—reduce the number of animals used in an experiment
- *Refinement*—minimize the suffering or pain of the animals still used in experiments

Although these principles were published more than 50 years ago, this topic is now more relevant than ever. In a digestibility experiment, a high number of broiler chickens is necessary for mainly two reasons. First, a certain number of birds per treatment are needed to obtain enough sample quantities for analyses. Due to the size of the growing animals, digesta samples from a single animal are often not sufficient for all chemical analyses. Therefore, it is common practice to pool samples of more than one animal per replicate (or pen). The WPSA proposed in its protocol (WPSA, 2013) a minimum number of eight birds per replicate. A certain number of replicates per treatment is essential for the proper evaluation and the statistical power of an experiment. Thus, the WPSA proposed a minimum of six replicates per treatment to obtain a representative result of the treatment (WPSA, 2013). Fewer replicates, in the worst case,

could lead to doubt cast upon the whole experiment. Efforts in helping to reduce the number of experimental animals are therefore highly welcome. Moreover, *in vivo* experiments are expensive and time-consuming. This is why our aim was to establish an *in vitro* assay, by which the digestive tract of a bird can be simulated specifically with regard to InsP degradation. The principles of the assay, advantages, and limitations are discussed herein.

3.2.1 General procedure and aim of the established *in vitro* assay

According to Longland (1991), a reliable *in vitro* system simulating digestive enzyme activity should consider the following aspects:

- The use of digestive enzymes in physiological amounts, considering for example the age of the simulated animal
- Adjustment of an adequate pH and consideration of the addition of relevant co-factors, co-enzymes, etc.
- Removal of the digestion products (simulation of absorption) to separate digested from undigested components and minimize end-product inhibition
- Adequate mixing in all digestion stages and physiological incubation durations

Several *in vitro* assays to simulate the digestive tract of poultry already exist for different research questions. They comprise either two steps to simulate the stomachs and small intestine (Bedford and Classen, 1993; Walk *et al*., 2012b; Yegani *et al*., 2013), or three steps with the crop as first step (Losada *et al*., 2009; Żyła *et al*., 1995). Morgan *et al*. (2014a) evaluated *in vitro* and *in situ* Ca and P solubility in two different protein sources with and without phytase supplementation for broiler chickens. Both methods detected interactions between the respective step, protein source, and phytase inclusion level. Their conclusion was that the assay used (Walk *et al*., 2012b) is suitable for evaluating the phytase effects in the stomachs and small intestine. The assay described by Żyła *et al*. (1995, 1999) was the basis for the *in vitro* assay in MANUSCRIPT 1. Żyła *et al*. (1995) established an assay by taking the following conditions into account:

- Matrix — Complete mixed feed or feed component
- Retention time — 30 min for crop, 45 min for stomachs, 60 min for duodenum
- pH — 5.8 for crop, 2.8 for stomachs, 6.1 for duodenum
- Temperature — 40 °C during all incubation steps

- Water content Increasing dilution
- Digestive enzymes Pepsin in stomachs, pancreatin in duodenum

The pH values were obtained in a previous *in vivo* experiment by Żyła *et al*. (1995). The absorption of end products was achieved through the use of dialysis tubes during the last incubation step.

3.2.2 Advantages of the established *in vitro* assay

In vitro assays simulating the digestive tract of animals, such as the one described in MANUSCRIPT 1, are generally faster and less expensive than *in vivo* experiments. A high sample throughput is possible. The InsPs can be extracted right after terminated incubation in the same centrifuge tube that was used for incubation. This reduces the time span in which added enzymes could theoretically continue to be active. The researcher is able to respond flexibly to outcomes and adjust the assay or parts of it quickly and in an uncomplicated way, which would not be possible in an *in vivo* experiment. Provided that the reliability of the assay can be proved, the number of *in vivo* experiments may be reduced. It is not possible to completely replace *in vivo* experiments, as described later. However, used as a pre-screening tool, it may help to reduce the number of treatments in an *in vivo* experiment and thus reduce the number of animals used.

The *in vitro* assay was established for the particular purpose of investigating the differences between phytases under standardized conditions and not as a digestion simulation in general. With the assay, it is possible to work without interference of factors varying between individual animals. There is no influence of endogenous phytases and phosphatases produced by epithelial cells or by microorganisms residing in the digestive tract. The processes of this assay are influenced only by the intrinsic plant phytase or added phytase products. The results obtained in this assay can thus be traced back to the interactions between the feed matrix and the supplemented enzyme product under the standardized conditions in the assay. Moreover, as the microbiota differs for each animal (Borda-Molina *et al*., 2016), there is no interindividual variation in the *in vitro* results.

The *in vitro* assay is carried out with a feed matrix instead of purified Na-phytate, which is used as phytate source in classical phytase assays (method 27.1.2) (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten [VDLUFA], 2007). Brejnholt *et*

al. (2011) incubated different phytate sources and feed materials with different phytases. They concluded that InsP degradation depends on the matrix that surrounds the phytate. As the phytate's surrounding matrix in purified Na-phytate differs from the phytate matrix in common feedstuff, the use of feed as matrix for evaluating feed enzymes *in vitro* was deemed to be a logical step.

3.2.3 Limitations of the established *in vitro* assay

Phytase concentrations

It was shown during the establishment of the assay described in MANUSCRIPT 1 that microbial phytase concentrations of 500 or more FTU/kg led to a very high $InsP_6$ disappearance when corn soybean meal-based feed was incubated over all three steps. Therefore, the effect of supplementation of increasing phytase levels to poultry feed was investigated. Six phytase concentrations (0, 100, 200, 300, 400, and 500 FTU/kg) were applied and the $InsP_6$ disappearance after incubation in all three steps was measured. The outcome, shown in FIGURE 3, revealed a linear relationship between phytase concentration and $InsP_6$ disappearance. According to the linear equation, 100% $InsP_6$ disappearance would be achieved with 586 FTU/kg. The experimental diets used for the *in vivo* experiment in MANUSCRIPT 2 were also incubated in the *in vitro* assay described in MANUSCRIPT 1. The results, presented in TABLE 1, show that the inclusion of calculated 1,500 FTU phytase/kg led to an almost complete disappearance of $InsP_6$ and $InsP_5$. However, between 0.2 and 0.4 µmol $InsP_6$/g DM and 0.1 and 0.3 µmol $InsP_5$/g DM were measured in treatments P+Ca-Phy+ and P+Ca+Phy+, suggesting a certain degree of end-product inhibition. Further, high concentrations of Ins(1,2,5,6)P_4, the main $InsP_4$ isomer produced by the added phytase (Zeller *et al*., 2015b), was found.

Two conclusions can be drawn from this experiment. First, in this *in vitro* assay, one cannot apply the same phytase concentrations as used in *in vivo* experiments. Second, the assay reacts in a plausible way by producing the $InsP_4$ isomer known as the main $InsP_4$ isomer of this specific phytase. Further, despite the high phytase level and the high degradation rate of $InsP_6$ and $InsP_5$, the diminishing effects of a P or Ca supplementation—as seen *in vitro* in MANUSCRIPT 1 and *in vivo* in MANUSCRIPT 2—are still observable.

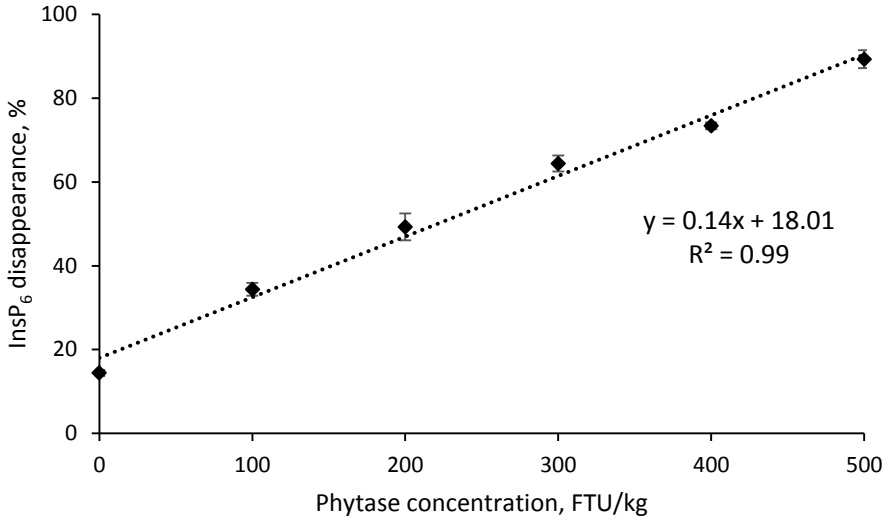

FIGURE 3. $InsP_6$ disappearance after incubation of a corn soybean meal-based diet (12.7 µmol $InsP_6$/g DM in complete diet) with graded levels of a supplemented microbial 6-phytase in the three-step *in vitro* assay. Each dot represents the mean ± standard error of at least three replicates. This experiment was carried out during the establishment of the *in vitro* assay described in MANUSCRIPT 1.

TABLE 1. Interaction of P, Ca, and phytase on concentrations of $InsP_{3-6}$ (µmol/g DM) in the experimental diets after incubation in a three-step *in vitro* assay simulating the crop, stomachs and small intestine of broiler chickens[1]. This experiment was carried out during Experiment 3 of MANUSCRIPT 1.

	Treatment[2]								pooled SEM	*P*-value[3]					
	P−Ca−		P−Ca+		P+Ca−		P+Ca+			P	Ca	Phy	P*Ca	P*Phy	Ca*Phy
	Phy−	Phy+	Phy−	Phy+	Phy−	Phy+	Phy−	Phy+							
$InsP_6$	14.8	0.2	14.7	<LOQ[4]	14.6	0.2	14.6	0.4	0.12	0.340	0.515	<0.001	0.594	0.443	0.576
$InsP(1,2,4,5,6)_5$	1.1	n.d.[5]	1.1	n.d.	1.0	0.1	1.1	0.3	0.04	0.717	0.139	<0.001	0.290	.	0.767
$InsP(1,2,3,4,5)_5$	0.6	n.d.	0.7	n.d.	0.5	n.d.	0.5	<LOQ	0.04	0.040	0.438	.	0.438	.	.
$InsP(1,2,3,4,6)_5$	<LOQ	n.d.	0.3	n.d.	<LOQ	n.d.	<LOQ	n.d.	0.00	.	.	.	.	.	.
$Ins(1,2,5,6)P_4$	n.d.	7.4	<LOQ	9.1	n.d.	11.3	n.d.	12.6	0.20	<0.001	<0.001	.	0.296	.	.
$InsP_3$[6]	n.d.	3.9	n.d.	4.1	n.d.	4.1	n.d.	3.6	0.10	0.365	0.148	.	0.008	.	.

[1]Data are given as treatment means with respective standard error of the mean (SEM); n=3

[2]Calculated composition: P−, 4.1 g P/kg DM; P+, 6.9 g P/kg DM; Ca−, 6.2 g Ca/kg DM: Ca+, 10.3 g Ca/kg DM; Phy−, 0 FTU/kg; Phy+, 1,500 FTU/kg

[3]As the three-way interaction could not be estimated, only the two-way interactions and main effects are displayed

[4]<LOQ, not quantifiable in the majority of samples

[5]n.d., not detectable in the majority of samples

[6]At least one of the following isomers: $Ins(1,2,6)P_3$, $Ins(1,4,5)P_3$, $Ins(2,4,5)P_3$

Simulation of the digestive tract

Longland (1991) stated that "[…] it is clear that the digestion of feeds by pigs and poultry is a highly dynamic, integrated process, under hormonal and neural control, which responds to a number of stimuli. To simulate such a complex system in its entirety using a comparatively static, unresponsive *in vitro* method would be extremely difficult, and would be unlikely to be cheap, quick or easy to perform or to allow many samples to be analysed simultaneously —criteria often set for the adoption of *in vitro* methods." Minekus *et al*. (1995), however, accepted the challenge to meet all five aspects proposed by Longland (1991). They developed a computer-controlled multicompartmental dynamic model simulating the human digestive tract segments. In this model, it is possible to simulate the gastrointestinal peristalsis; thus, the transport mechanism, and a proper mixing of the digesta is ensured. The transit time may be varied. Further, pH and bile salt concentrations are controlled by a computer and based on *in vivo* data. Absorption processes are also enabled by using dialysis membranes. This highly complex system is of course more accurate than the assay that was established in the present work. However, it does not meet the requirements of analyzing many samples, such as short time, easy handling, or cost efficiency. We did not claim to mimic the digestive tract as closely as possible; rather, our intention was to establish an *in vitro* assay for a specific purpose—the investigation of differences between feed supplements in an *in vitro* assay, which should be more suitable than the classical phytase assays with purified Na-phytate carried out at only one pH value (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten [VDLUFA], 2007). Therefore, we decided to establish an *in vitro* assay that is easy to handle, brings fast results, and has high reproducibility. It is clear that this departs from the dynamic processes in a living animal, but the focus was on a high standardization, in particular for the comparison of results. We adapted the specifications of Żyła *et al*. (1999) and decided not to vary the pH, retention time, or other variables. However, it would be possible to refine or change the assay and set the conditions closer to the conditions in the animal for the investigation of other questions. For example, phosphatases could be added to Step 3 (simulation of small intestine) to investigate whether they are able to further degrade the accumulated $InsP_{3-4}$ isomers. It is also possible to vary the pH in all three steps, depending on the incubated feed. Therefore, pH measurements in the digesta of freshly sacrificed birds fed with different kind of feed would be necessary. Incubating fresh

material from the mucosa or digesta of birds to bring endogenous phytases and phosphatases into the system would also be worth trying. However, this would depart from the ease, reproducibility, and animal-friendliness of the established assay. One point that we did not adapt from Żyła *et al*. (1999) is the simulation of absorption in the small intestine, which they achieved by the application of dialysis tubing. The molecular weights of $InsP_{1-6}$ (260–660 Dalton) and P (31 Dalton) (Cooper *et al*., 2007) are too close to each other to be separated by a dialysis membrane. According to a supplier of dialysis membranes (Carl Roth GmbH, Karlsruhe), the molecules to be separated should have a size ratio of 25:1, which is not the case with InsPs and P. Thus, the end product P cannot be removed from the incubated medium without removing InsPs as well. InsPs, however, should stay in the incubated medium to be further available to the added feed enzyme. Due to the inability to remove only P, an end-product inhibition may occur.

Significance of InsP concentrations *in vitro* and comparability to *in vivo* data

Apart from the effect of phytase supplementations above 500 FTU/kg, TABLE 1 reveals that the concentrations of InsP isomers measured *in vitro* differ from those measured *in vivo* (MANUSCRIPT 2). Pontoppidan *et al*. (2007) incubated a *Peniophora lycii* phytase supplemented soybean meal corn-blend in an *in vitro* simulation of the digestive tract of pigs. Most of $InsP_6$ had been disappeared during the second step (simulation of the stomach) which is in agreement with our data. No further InsP degradation occurred in the simulation of the small intestine (pH 6.8–7.0). As the pH prevailing in this step is above the pH optimum for the applied phytase, this finding is not surprising. *In vivo*, further degradation takes place due to endogenous microbial and epithelial phosphatases with optima at pH values prevailing in the small intestine. It is apparent that the processes occurring inside the bird are far more complex than can be simulated *in vitro*. This was shown by the appearance of $Ins(1,2,3,4)P_4$ in the treatments without added phytase *in vivo*, but not *in vitro*. Moreover, the added phytase managed to degrade almost all $InsP_6$ and $InsP_5$ *in vitro*, but must have been affected by several diminishing factors *in vivo*, because 100% $InsP_6$ disappearance has not been detected so far *in vivo*. Also, it seems that $Ins(1,2,5,6)P_4$ and isomers of $InsP_3$ accumulate in the *in vitro* assay. On the one hand, this particular phytase might have problems in degrading this $InsP_4$ isomer. On the other hand, the accumulation might have also been caused by the missing absorption step leading to the inhibition of the phytase by the end product P. The inhibiting effect of

nutrients when no dialysis step is implemented was shown by Lan *et al*. (2010) who compared the *in vitro* assays of Tervilä-Wilo *et al*. (1996) and Żyła *et al*. (1995). The former simulated three steps of digestion (crop, stomachs, and small intestine), the latter also comprised the three steps, but implemented the aforementioned dialysis step during the third step. The feed-to-liquid ratios, pH values, temperature, and addition of digestive enzymes were identical in the assays of the two studies. The only differences were the use of an acetate buffer in the assay of Tervilä-Wilo *et al*. (1996), which was not applied in the assay of Żyła *et al*. (1995) and a longer incubation time for Step 3 of the assay of Żyła *et al*. (1995). Lan *et al*. (2010) reported that P was released with increasing phytase supplementation in a quadratic manner. Moreover, more P was released using the method of Żyła *et al*. (1995), which they explained with the absorption process. The released P may have passed the dialysis membrane and no longer acted as an end-product inhibitor of the applied phytase. However, InsPs should have also been able to pass the dialysis membrane and therefore no longer be available for the phytase as well. Thus, the question remains open as to whether the dialysis step really contributed to a greater P release. Further, Lan *et al*. (2010) validated their results with the *in vivo* data of broiler chickens. The total tract apparent digestibility of P, CP, DM and AME *in vivo* could be predicted with the *in vitro* P release. Positive correlations between *in vitro* P release and growth performance and P digestibility of growing pigs fed P-deficient diets were also found in the study by Liu *et al*. (1997). However, this cannot be transferred to studies investigating InsP degradation. But it became obvious that the InsP isomer patterns observed for the added phytase *in vitro* and *in vivo* were similar. Pontoppidan *et al*. (2012) studied the degradation pathway of a *C. braakii* phytase *in vitro* and *in vivo* in piglets. As in MANUSCRIPT 1, they reported that the InsP isomers formed by the applied phytase were the same *in vitro* and *in vivo*. They also reported an accumulation of the respective $InsP_4$ isomer *in vitro* but not *in vivo*.

It is not possible to completely replace an *in vivo* experiment with an *in vitro* assay as described in MANUSCRIPT 1 or as used by others (Morgan *et al*., 2014a). Menezes-Blackburn *et al*. (2015), in their work comparing different phytase products in a three-step *in vitro* assay, also concluded that it is not suitable to investigate the bioefficacy of phytases but to rank feed enzymes for their potential use in poultry feed. It can be used as a pre-screening tool for feed enzymes which can then be selected for further *in vivo* experiments. Unnecessary *in vivo*

experiments might be prevented and consequently the number of animals reduced. Thus, the *in vitro* assay established during this work is a contribution to the principle of the three Rs—in particular to the point *Reduction*.

3.3 Dietary effects on $InsP_6$ disappearance and InsP degradation products *in vivo*

Based on the findings of Shastak *et al*. (2014) and Zeller *et al*. (2015c) the question arose whether the observed diminishing effects of the supplemented MCP on $InsP_6$ disappearance and the appearance of lower InsP esters arose from the supplemented P or Ca or both. This question was investigated in two experiments. In the first experiment, the effects of P, Ca, and phytase on the (dis)appearance of InsPs were studied in the *in vitro* assay described in Chapter 3.2. In the second experiment, the single or interactive effects were investigated in an *in vivo* experiment. The effect of phytases—especially of the phytase used in the experiments of this thesis—on $InsP_6$ disappearance and the appearance of lower InsP isomers has already been discussed in the works of Zeller (2015). Thus, the focus of the present thesis is on the effects of dietary P and Ca. Amongst the plethora of possible influencing factors on $InsP_6$ disappearance that would go beyond the scope of this thesis, the discussion is limited to some major points.

Further, the effects of P, Ca, and phytase on the concentration of MI in the digestive tract and blood (Chapter 3.4) and the prececal digestibility of AAs (Chapter 3.5) are discussed separately.

3.3.1 $InsP_6$ disappearance

Crop

$InsP_6$ disappearance in the crop was 30% in the control treatment without added phytase in the experiment of MANUSCRIPT 3. In the experiment of MANUSCRIPT 2 about 2–12% of the $InsP_6$ disappeared in the crop. This difference can be explained by the inclusion of wheat as the main component in the experiment of MANUSCRIPT 3, whereas the main components in the experiment of MANUSCRIPT 2 were soybean meal and corn. The intrinsic phytase activity is high in wheat but negligible in corn or soybeans (Eeckhout and de Paepe, 1994; Rodehutscord *et al*., 2016). Compared to the $InsP_6$ disappearance of 59% in the crop of broilers that were also fed a wheat-based diet in the experiment by Zeller *et al*. (2016), the observed $InsP_6$ disappearance in the crop in MANUSCRIPT 3 (30%) was clearly lower. This may have two reasons.

First, the experiment by Zeller *et al*. (2016) had a higher inclusion level of wheat, and thus eventually a higher phytase activity in the complete diet. Second, the level of P and Ca was reduced in the experiment by Zeller *et al*. (2016), whereas in the present study, the P and Ca concentrations were at the recommended level. Phosphorus and Ca supplementations reduced $InsP_6$ disappearance, in particular if both P and Ca were supplemented, as shown in MANUSCRIPT 2. This may also be the reason $InsP_6$ did not further disappear up to the terminal ileum (31%; MANUSCRIPT 3), whereas it reached 69% up to the terminal ileum in the experiment by Zeller *et al*. (2015a). However, a possible higher $InsP_6$ disappearance up to the terminal ileum due to higher wheat inclusion can be ruled out, as the experiment by Zeller *et al*. (2015a) with untreated wheat and microwave-treated wheat (deactivated phytase) shows that the intrinsic wheat phytase no longer plays a role in the small intestine. Possibly, the wheat phytase is inactivated during the passage through the stomachs and the anterior parts of the small intestine, as Phillippy (1999) showed that wheat phytase is sensitive to pepsin and pancreatin. Further, Shastak *et al*. (2014) reported a decrease in prececal $InsP_6$ disappearance and an increase of the concentrations of $InsP_5$ isomers in the ileum with the supplementation of MCP (from 3.0 to 4.6 g total P/kg respectively), irrespective of whether the diet was corn- or wheat-based. These findings indicate that the intrinsic wheat phytase is only relevant in the anterior segments of the digestive tract and of no further benefit during the passage through the small intestine.

In the absence of the microbial phytase, the $InsP_6$ disappearance in the crop was significantly higher in treatments with further added Ca than in treatments without further Ca supplementation, as described in MANUSCRIPT 2. The microbial population in the crop mainly consists of Lactobacillaceae (Borda-Molina *et al*., 2016) and phytase and phosphatase activities were found in different *Lactobacillus* strains (Haros *et al*., 2008). Craven and Williams (1998) demonstrated that the attachment of *Lactobacilli* to mucus is enhanced by Ca. Further, Choi *et al*. (2001) reported that a phytase from *Bacillus* sp. KHU-10 requires Ca for its activity and stability. They suggested that Ca can help to maintain the active conformation of the enzyme, which is not possible with other metal ions. Phytases from *Bacillus amyloliquefacience* are also Ca^{2+}-dependent (Oh *et al*., 2001). Tamim *et al*. (2004), in an *in vitro* experiment, found that incubating a 3-phytase of fungal origin increased P release from Na-phytate with increasing Ca concentrations at pH 2.5. At pH 6.5, this effect was reversed.

Therefore, it is potentially possible that phytases produced by some microorganisms in the crop need Ca ions for their activity. The increased $InsP_6$ disappearance in the crop due to Ca supplementation may also have been caused by a change in the pH toward more favorable conditions for the phytase-producing microbiota in the crop. However, pH was not measured in this experiment. This should be considered in future experiments.

Small intestine

Effects of dietary P and Ca in the absence of supplemented microbial phytase

The results of the experiment presented in MANUSCRIPT 2 indicate a decreasing effect of the P supplementation on $InsP_6$ disappearance up to the terminal ileum when no phytase was present. It was further decreased by the additional supplementation of Ca. The diminishing effect of P was also supported by the observation of a less pronounced increase of $InsP_6$ disappearance between duodenum+jejunum (together) and the terminal ileum in both P-added treatments (4 and 5 percentage points in P+Ca− and P+Ca+, respectively) than in the other treatments without added P and without phytase (10 and 15 percentage points in P−Ca− and P−Ca+, respectively). The observed effects of dietary P and Ca in treatments without phytase supplementation were not observed *in vitro*, as can be seen in MANUSCRIPT 1 and TABLE 1. The $InsP_6$ disappearance *in vitro* was too low to observe any treatment effects, possibly due to the lack of endogenous epithelial and microbial phytases and phosphatases. This in turn shows that the diminishing effects of P and Ca observed *in vivo* are likely to be effects on the endogenous mucosal or microbial phytases and phosphatases in diets with no added phytase.

Shastak *et al*. (2014) reported a decreasing total tract $InsP_6$ disappearance and increasing $InsP_5$ isomer concentrations in the excreta with increasing dietary P by the supplementation of monosodium phosphate to a P-reduced corn soybean meal-based diet (from 3.1 to 4.4 g total P/kg feed). However, to maintain a constant Ca:P ratio, Ca was supplemented as well. Thus, the effects observed cannot be ascribed solely to the supplemented P. The diminishing effect of P supplementation when no phytase was present, as observed in MANUSCRIPT 2, could have been caused by P end-product inhibition on the endogenous phosphatases present in the small intestine (Zeller *et al*., 2015c). Greiner *et al*. (1993) demonstrated *in vitro* an inhibition of two *E. coli* phytases by phosphate. Several studies reported an increased activity of phytase

or phosphatases in the mucosa of chickens due to P deficiency (Abudabos, 2012; Huber *et al*., 2015; McCuaig *et al*., 1972). However, $InsP_6$ disappearance up to the ileum was significantly increased with 0.45% NPP instead of 0.28% NPP across two Ca levels in the study by Li *et al*. (2016), while Ballam *et al*. (1985) reported an increased PP hydrolysis in excreta with increasing NPP (0.12 vs. 0.45%) at a low Ca level but not at a high Ca level (0.09 vs. 1.0%) in the diet. Why these results contradict the results of the present experiment (MANUSCRIPT 2) cannot be assessed at this time.

Calcium supplementation alone did not have any effect on $InsP_6$ disappearance up to the terminal ileum when no phytase was added (MANUSCRIPT 2). In contrast, Ballam *et al*. (1985) found decreased phytate hydrolysis in excreta of three-week-old broiler chickens with increased dietary Ca (0.09 to 1.0% Ca) at two NPP levels (0.12 and 0.45% NPP). Interestingly, they also observed 8.3% and 24.5% phytate hydrolysis in two consecutive experiments with the same diet (0.12% NPP and 1.0% Ca). The reason remains unknown but this may have been the first hint toward the great variation in P-digestibility experiments, as described by Rodehutscord *et al*. (2017). Tamim *et al*. (2004) also reported a marked decrease in PP disappearance up to the whole ileum (69 vs. 25%) of 24-day-old broilers when dietary Ca was increased from 0.2 to 0.7% (with 0.41% P). The studies by Tamim *et al*. (2004) and Ballam *et al*. (1985) do not necessarily have to be contradictory to the results of MANUSCRIPT 2, where no effect of Ca addition alone was found. The Ca concentrations vary widely between MANUSCRIPT 2 and the experiments by Ballam *et al*. (1985) and Tamim *et al*. (2004), with ranges from almost no dietary Ca to a high Ca level in the latter ones. Amerah *et al*. (2014) reported phytate degradation rates of 51, 40, 44, and 40% with increasing Ca concentrations (0.4, 0.6, 0.8, 1.0%) and 0.51% P without added phytase in 21-day-old broilers fed corn-based diets. The results of the study by Amerah *et al*. (2014) are in agreement with the present results when the applied Ca concentrations are compared. In the study by Amerah *et al*. (2014), there was no difference in phytate degradation between 0.6 and 0.8% Ca. Accordingly, Plumstead *et al*. (2008) reported that the decreasing effect of Ca supplementation on prececal PP-digestibility in broilers was not significant at a Ca level above 0.7%.

Ballam *et al*. (1984) reported that 1.0% Ca and 0.5% NPP in the diet decreased phytate hydrolysis compared to 0.85% Ca and 0.42% NPP over a range of different fiber and phytate sources in the excreta of three-week-old female broiler chickens. Delezie *et al*. (2012) also

found higher P release from PP in diets with low P and Ca and in diets with standard Ca and low P than in diets with standard Ca and P without phytase in the excreta of three-week-old broilers. These studies confirm the outcome of the experiment in MANUSCRIPT 2 where the supplementation of P and Ca combined led to the significantly lowest $InsP_6$ disappearance up to the terminal ileum in the absence of added microbial phytase. A possible explanation might be as follows: The diminishing effect of the P supplementation led to higher $InsP_6$ concentration in the digesta and consequently to decreased $InsP_6$ disappearance. This may have led to a subsequent complexation of $InsP_6$ with the added Ca, which in turn led to an even greater decrease in $InsP_6$ disappearance.

The study by McCuaig *et al*. (1972) reported that excess Ca markedly decreased the activity of both alkaline phosphatase and phytase in duodenal mucosa of chickens. In contrast, Hanna *et al*. (1979) found a stimulatory effect of Ca. They investigated the effects on alkaline phosphatases of isolated brush border and basal lateral membranes of rat duodenal epithelium. This was supported by those of Brun *et al*. (2012), who reported an increased activity of intestinal alkaline phosphatase, resulting in a decrease in luminal pH and Ca absorption (Brun *et al*., 2014) in rat duodenum after increasing dietary Ca but not an increase in the expression of intestinal alkaline phosphatase. Interestingly, Applegate *et al*. (2003) reported a decrease in the activity of intestinal phytase in purified brush-border vesicles in two of three experiments due to increased dietary Ca levels, whereas Ca supplementation increased the activity in the third experiment. The reason for this difference remains open.

The microbial population in the digestive tract is also known to be influenced by dietary P and Ca or pH changes (Heyer *et al*., 2015). In the ileum digesta of broilers fed a diet based on wheat and soybean meal with supplemented Ca and P, Ptak *et al*. (2015) found decreased lactate-producing bacterial groups and an increase in *Clostridium perfringens* and Enterobacteriaceae. Borda-Molina *et al*. (2016) reported changes in the microbial community in different segments of the digestive tract of broilers of the same experiment as described in MANUSCRIPT 2, due to varying P, Ca and phytase levels in the diets. However, no consistent pattern was found throughout the whole digestive tract. A high variation in the microbial community of different digestive tract segments of individual birds could eventually have masked potential dietary effects. An effect of MCP supplementation on the bacterial community composition in the crop but not in the ileum of 25-day-old broiler chickens fed a corn and soybean meal-based

diet was detected by terminal restriction fragment length polymorphism (T-RFLP) analysis in the study by Witzig *et al*. (2015).

However, it is not possible for now to quantify the contribution of epithelial cells or the microbiota to degrade InsPs. Thus, it also cannot be distinguished if the different treatments primarily affected the epithelial or microbial enzyme production. This would be possible with an experiment using germfree chickens, where at least the contribution of the epithelial cells can be investigated.

It seems likely that dietary P and Ca affect the $InsP_6$ degradation in the small intestine through the (i) regulation of mucosal phosphatases, (ii) change in the pH, (iii) complexation of phytate, and (iv) a change in the microbial population. However, the effects of dietary P and Ca are not consistent across experiments and may therefore be dependent on, among other factors, the concentrations and source of P, phytate, and Ca, and the age of animals.

Effects of dietary P and Ca in the presence of supplemented microbial phytase

When phytase was supplemented, there was a numerical but not significant decrease in $InsP_6$ disappearance up to the terminal ileum in the Ca supplemented treatments (MANUSCRIPT 2). The response in growth performance to phytase supplementation was greatest in the treatment P−Ca+ and explainable by a higher P provision due to phytase supplementation. However, growth performance did not reach the level of the P−Ca− diet supplemented with phytase or the P supplemented treatments. This, together with the numerically decreased $InsP_6$ disappearance up to the terminal ileum, indicates that Ca does have an effect per se on the phytase or $InsP_6$ degradation. It is likely that the Ca effect derived from Ca-phytate complexes, caused by increasing pH and higher Ca concentrations in the small intestine. Pontoppidan *et al*. (2007) investigated Ca effects on a *Peniophora lycii* phytase incubated with a soybean meal corn-blend in an *in vitro* pig digestion assay. At a very low Ca level (1.2 g/kg DM), $InsP_6$ disappeared completely at pH 5.0 with increasing concentrations of $InsP_{3-5}$. Increasing the Ca concentration to 6.2 g/kg DM led to a decreased $InsP_6$ disappearance during the same phase. However, $InsP_{4-5}$ did not accumulate. The authors explained this finding with the preference of the phytase to degrade $InsP_6$ before degrading lower InsP esters. However, at higher Ca concentrations, the formation of phytate-Ca complexes led to a decreased phytate solubility, which, in turn, led to an increased degradation of lower InsP esters, as $InsP_6$

was not available to the full extent. This leads to the conclusion that Ca does not have an effect on phytases per se, but reduces the phytate degradation by forming Ca-phytate complexes.

The results of MANUSCRIPT 2 regarding P disappearance, which was obviously decreased by the addition of Ca, suggest that formation of Ca-phosphate occurred in the small intestine making P unavailable for absorption. The numerically lower $InsP_6$ disappearance up to the small intestine in the Ca treatments with phytase further suggests that the formation of Ca-phytate complexes made the phytate less susceptible to phytase and the Ca less available for absorption. Appropriately, the amount of disappeared Ca up to the terminal ileum was lower in P+Ca+Phy+ than in P−Ca+Phy+, whereby the addition of only P had no effect. Less disappeared Ca means more Ca in the digesta which may have led to even less digested P due to Ca-phosphate complexes.

Ca:P ratio

The $InsP_6$ disappearance up to the terminal ileum in the absence of added microbial phytase was lowest in treatment P+Ca+ and highest in treatment P−Ca−, although both had the same Ca:P ratio. The phytase-supplemented treatments did not differ significantly from each other, although Ca:P ratios ranged from 0.8 to 2.3. Driver *et al*. (2005) reported that most studies investigating the efficacy of phytases concluded that high Ca:P ratios decrease phytase efficacy. Driver *et al*. (2005) further argued that these studies are somewhat misleading, as the greatest increase in broilers performance was always observed in treatments with high or standard Ca levels and reduced P levels indicating that the efficiency of phytase is highest in these treatments due to an increased provision with P and thus a reduced P deficiency. This was the case in the experiment presented in MANUSCRIPT 2. The greatest improvement in all performance traits due to phytase supplementation occurred in treatment P−Ca+ whereby the level of the P or P and phytase-supplemented treatments has not been reached. The improvement due to phytase supplementation was highest in the treatment with highest Ca:P ratio, but the total performance was still the lowest. This can also be applied to $InsP_6$ disappearance, whereby the effects of the minerals were slightly different. The greatest improvement by phytase was achieved in treatment P+Ca+, having the same Ca:P ratio as P−Ca−. However, the diminishing effects of P+Ca+ were still seen on the level of lower InsP esters. The statement of Driver *et al*. (2005) was confirmed by a meta-analysis investigating dietary NPP and Ca concentrations for optimal growth (Létourneau-Montminy *et al*., 2010).

They reported that a maximum average daily gain was achieved with a Ca:P ratio of 2.3 in a diet with 4.4 g NPP/kg and with a Ca:P ratio of 1.5 in a diet with 4.0 g NPP/kg. Thus, especially in studies investigating Ca and P effects on different traits, it is not only the Ca:P ratio that should be discussed but also total amounts of Ca and P in the diet.

Ca source and particle size

In literature, the outcomes of studies investigating the effects of Ca supplementations on $InsP_6$ disappearance, P digestibility and epithelial phytase activity are not consistent, as shown in the previous paragraphs. The diets used in digestibility experiments vary in not only the concentration but also the source of nutrients. The properties of nutrients may vary depending on source, charge, and particle size. Limestone is quite commonly used as a Ca source due to its cost-efficiency (Anwar and Ravindran, 2016). However, Anwar *et al*. (2016a) reported Ca concentrations varying between 390 and 420 g/kg in three analyzed commercially available limestone sources. Also, the mineral composition of these samples varied. The prececal digestibility of Ca in treatments with either of these samples varied between 54 and 61%. Differences due to particle size were ruled out as the samples were ground to pass a 0.2 mm sieve. Further, Anwar *et al*. (2016b) reported lower apparent and true (corrected for endogenous losses) ileal Ca digestibility in broiler chickens using limestone with a particle size of < 0.5 mm than with a particle size of 1.0–2.0 mm, which was supported by Anwar *et al*. (2017). Manangi and Coon (2007) found that the smallest particle size of limestone (28 µm) with the highest solubility gave indications for decreased $InsP_6$ disappearance *in vitro* and *in vivo*. Medium-to-fine particle sizes of several Ca sources led to a higher performance of broiler chickens than coarser particles (Guinotte and Nys, 1991; McNaughton *et al*., 1974). Walk *et al*. (2012a), in an experiment with 0.62% P and 0.9% Ca of a highly soluble calcified seaweed (HSC), found lower body weight gain, tibia ash weights and reduced P digestibility compared to the treatment with 0.9% Ca of limestone (0.77% P). Paiva *et al*. (2013) reported a higher necrotic enteritis-associated mortality when 0.9% Ca of HSC—instead of 0.6% Ca of HSC or limestone—was implemented in the diets (0.76% P) of broiler chickens during a necrotic enteritis period. Walk *et al*. (2012a) and Paiva *et al*. (2013) concluded that HSC—as a more soluble Ca source—can be used in dietary inclusion levels lower than recommended levels for Ca, especially in combination with phytase supplementation. This was again supported by the studies by Bradbury *et al*. (2016a,b). Other studies investigating alternatives to limestone

report either an increased performance of broiler chickens due to the supplementation of Ca citrate-malate compared to limestone (Henry and Pesti, 2002) or no improvements in performance due to the inclusion of Ca citrate-malate, Ca citrate, marble, sea shell, or oyster shell compared to limestone (Augspurger and Baker, 2004; Guinotte and Nys, 1991; McNaughton *et al*., 1974). Hamdi *et al*. (2015) reported a change in the *in vitro* solubility of three Ca sources (tricalcium phosphate, Ca chloride, and limestone) depending on the pH. Also, the acid-binding capacity increased with increasing Ca concentration. The accompanying *in vivo* experiment of Hamdi *et al*. (2015) with Ca concentrations of 0.55% and varying NPP levels (0.30–0.45%) revealed that—in contrast to limestone or tricalcium phosphate—birds receiving Ca chloride had lower weight gain and added NPP did not increase tibia weight in the presence of Ca chloride. However, feed efficiency and the pH in the gizzard and proventriculus were not affected by Ca source.

These results, taken together, may explain to some extent the variation between results of studies using different Ca sources and emphasize the difficulty in comparing results from different studies.

In the context of Ca in poultry diets, the known effects of Ca on the availability of nutrients or on the pH in the digestive tract, and the varying availability of Ca depending on the Ca source, it is astonishing that diet formulations are still calculated with total Ca values (Angel, 2010; Proszkowiec-Weglarz and Angel, 2013). As limestone is mostly used as Ca source, is quite cheap, and is not a limited resource like P, there seems to be no need to change the valuation system for Ca (Anwar and Ravindran, 2016). Regarding the comparability of results, the avoidance of under- or oversupply of Ca, and the unavoidable link between Ca and P digestion, it would be beneficial to calculate with digestible Ca instead of total Ca. However, the change toward a new system is laborious and associated with great effort in developing methodologies that can be applied routinely by laboratories (Angel, 2011; Anwar and Ravindran, 2016).

3.3.2 Degradation steps between $InsP_6$ and *myo*-inositol in the ileum

The degradation pattern of InsPs in the digestive tract reveals additional information about the efficiency of added feed enzymes, inhibitory effects of other supplements and the

resulting provision with P for the bird. Therefore, the degradation pattern in dependence of different feed supplements in two segments of the digestive tract are discussed herein.

FIGURE 4 shows the relative proportions of $InsP_{3-6}$ esters and MI in the digesta of the duodenum+jejunum (a) and terminal ileum (b) of broiler chickens in the experiment described in MANUSCRIPT 2 depending on the dietary treatment. Although ileum digesta was also analyzed for $InsP_{1-2}$ esters, only $InsP_{3-6}$ were considered here for a better comparability and visualization of changes in the InsP pattern between the two digestive tract segments.

Interestingly, the largest proportion of the sum of InsPs and MI consisted of $InsP_6$ and MI. In the duodenum+jejunum, $InsP_6$ had a larger proportion than MI in the treatments without supplemented phytase, and vice versa in the treatments with added phytase. The proportion of $InsP_6$ of the sum of InsPs and MI in the duodenum+jejunum was generally as follows, independent of phytase addition: P−Ca− < P−Ca+ ≈ P+Ca− < P+Ca+. The proportion of $InsP_6$ of the sum of InsPs and MI increased whereas the proportion of MI decreased from duodenum+jejunum to the terminal ileum. In the treatments with supplemented phytase, the proportion of $InsP_6$ of the sum of InsPs and MI in the ileum was as follows: P−Ca− ≈ P+Ca− < P−Ca+ ≈ P+Ca+. These results have two indications. First, it seems that the supplemented minerals or the combinations of both acted differently in the two digestive tract segments. The effects observed were caused by either a change in the degradation of the InsPs or in the absorption of InsPs and MI or both. In the duodenum+jejunum, P and Ca alone seem to have exerted the same effect respectively. The combination of the two had an even larger effect on InsPs in the ileum independent of phytase addition resulting in a higher proportion of $InsP_6$ and a smaller proportion of MI of the sum of InsPs and MI compared to the other treatments. In the ileum, the addition of P seemed to have no effect and Ca alone or P and Ca together to have an effect on the InsPs when phytase was supplemented.

Zeller *et al*. (2015c) found identical $InsP_6$ disappearance up to the terminal ileum in corn soybean meal-based diets, whether or not supplemented with MCP, when a high phytase dose was applied (12,500 FTU/kg), but higher concentrations of Ins(1,2,5,6)P_4 and $InsP_3$ isomers in the presence of MCP, indicating a diminished degradation of lower InsP esters due to MCP. As the same was found *in vitro* (TABLE 1), where no endogenous phytases were present, one might suggest that the P and Ca supplementation either had a direct effect on the applied phytase or complexes between P, Ca, and lower InsP ester isomers occurred.

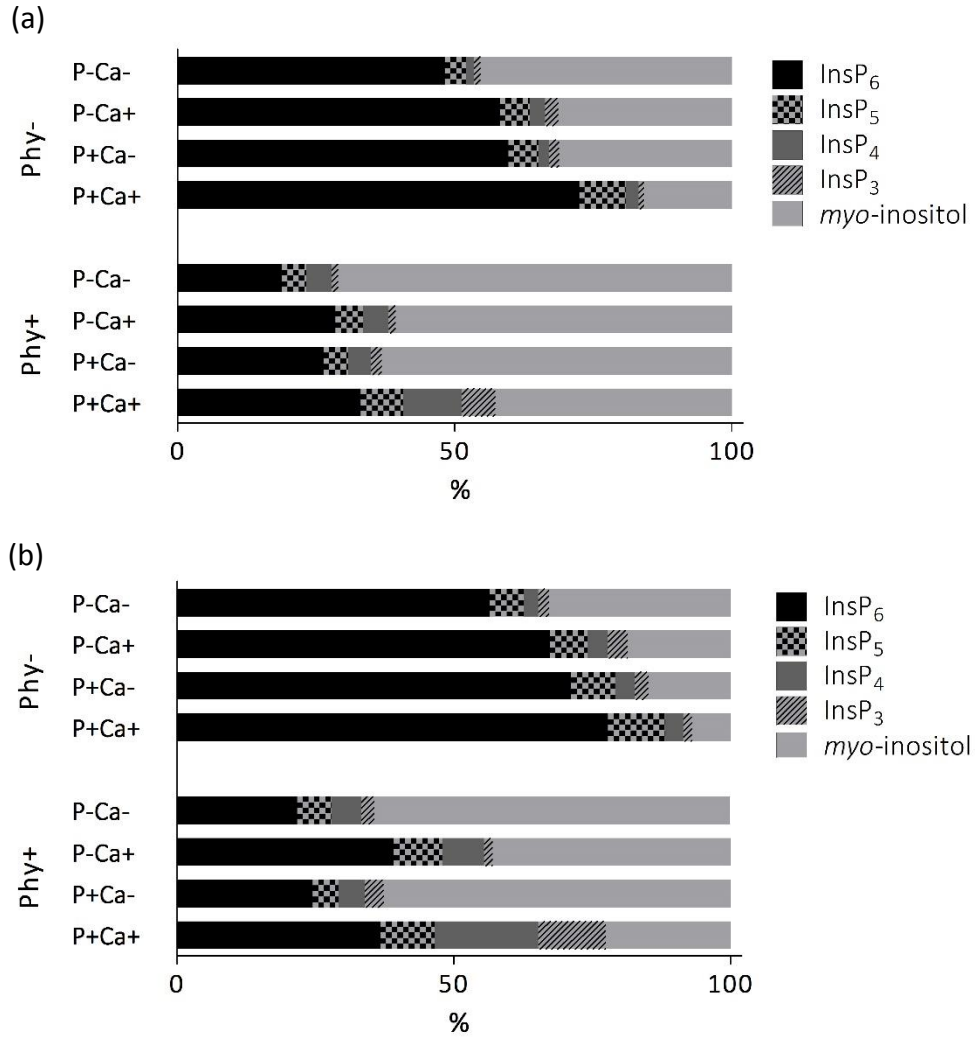

FIGURE 4. Relative proportions of the concentrations of $InsP_{3-6}$ esters and *myo*-inositol in the digesta of the duodenum+jejunum (a) and the terminal ileum (b) of 27-day-old broiler chickens depending on P, Ca and phytase supplementation. The sum of the concentrations of $InsP_{3-6}$ esters and *myo*-inositol on a molar basis is defined as 100%. Data derived from MANUSCRIPT 2.

However, $InsP_{1-4}$ have a lower affinity for proteins (Yu *et al.*, 2012) and metal ions (Persson *et al.*, 1998) than $InsP_{5-6}$, which makes the latter suggestion less relevant. Second, based on the finding that the proportion of MI decreased or remained unchanged between duodenum+jejunum and the terminal ileum—although it should increase due to further $InsP_6$ degradation and MI formation—it might be suggested that some of the MI was absorbed between duodenum+jejunum and ileum.

FIGURE 5 shows the measured concentrations of InsP esters, $InsP_{1-2}$ included, and MI (a) and the relative proportion of InsPs and MI (b) in the digesta of the terminal ileum. It is obvious that the sum of the measured concentrations of InsPs and MI were higher in the treatments without added phytase, whereby the following gradation was observed: P−Ca− ≈ P−Ca+ < P+Ca− < P+Ca+. This is in line with the results for the $InsP_6$ disappearance up to the terminal ileum (56%, 54%, 40%, and 21%, respectively). Looking at the relative proportions in FIGURE 5 (b), one can see that although the measured concentrations of InsPs and MI were almost identical in treatments P−Ca− and P−Ca+ without phytase (FIGURE 5 (a)), the proportions differed. Treatment P−Ca− had a lower proportion of $InsP_6$ and a higher proportion of MI of the sum of InsPs and MI than P−Ca+. When phytase was supplemented, the measured concentrations of the sum of InsPs and MI were the same for P−Ca−, P−Ca+, and P+Ca− and higher for P+Ca+. Although the proportion of $InsP_6$ in P+Ca+ was not higher than in treatment P−Ca+ with phytase addition (FIGURE 5 (b)), the proportion of MI was much lower; thus, the intermediate products, especially $InsP_4$, had a great proportion of InsPs and MI. Basically the same applied for treatments P−Ca− and P+Ca− with phytase supplementation. Despite the identical $InsP_6$ proportions, the proportion of intermediate products was higher in P+Ca− than in P−Ca−. These graphs impressively exemplify how the supplementation of phytase, P, or Ca and the combination of them changed not only the concentration of $InsP_6$ in the digesta, but also the pattern of all InsP esters and MI.

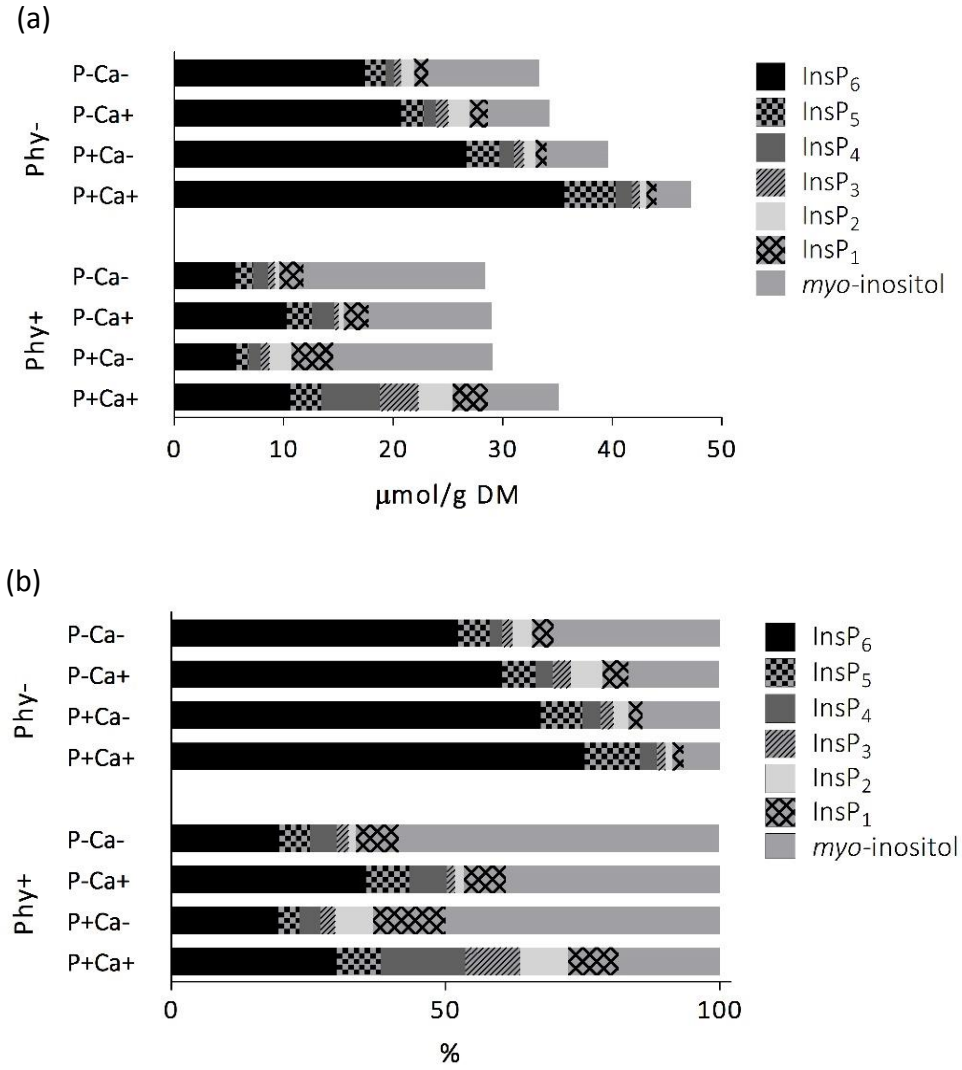

FIGURE 5. Measured concentrations (a) and relative proportions (b) of the concentrations of InsP esters and *myo*-inositol in the digesta of the terminal ileum of 27-day-old broiler chickens depending on P, Ca and phytase supplementation. Data derived from MANUSCRIPT 2.

3.3.3 Relevance of lower InsP esters for P evaluation

The degradation of lower InsP esters with consequent release of P can contribute to the P supply of the bird. In this context, Rodehutscord (2016) presented a graph where the digested P was plotted against the disappeared $InsP_6$-P. The underlying data were obtained in three experiments of his working group. He estimated by regression analysis that 0.78 g P was digested when 1 g $InsP_6$-P disappeared, concluding that a high proportion of $InsP_6$-P can be absorbed by birds. It was suggested that the 0.22 g of undigested P were due to an incomplete P release from $InsP_6$.

An attempt has now been made to calculate the proportion of P released from all InsP esters and subsequently digested by the birds. For the present calculation, it was taken into account that one molecule P is released and one molecule $InsP_5$ created per molecule disappeared $InsP_6$. For the calculation, data from the experiment described in MANUSCRIPT 2 were used as this was the only experiment in which all InsP esters ($InsP_{1-6}$) were analyzed. The following calculation steps were performed:

- Calculation of the potentially available P bound to InsPs in the feed by calculating the proportion of P on measured InsPs → $InsP\text{-}P_{feed}$
- To consider that nutrients can concentrate along the small intestine, the measured concentrations of all InsP esters in the ileum were multiplied with the titanium (Ti) (indigestible marker) factor ($Ti_{feed}/Ti_{digesta}$)
- Calculation of the amount of P that is bound to InsPs, corrected by using the Ti factor, in the ileum → $InsP\text{-}P_{digesta}$
- Calculation of the amount of P that is no longer bound to InsPs in the ileum, thus must have been released during the passage through the digestive tract. Calculated by the difference between $InsP\text{-}P_{feed}$ and $InsP\text{-}P_{digesta}$ → $InsP\text{-}P_{released}$
- Plotting $InsP\text{-}P_{released}$ against $P_{digested}$ in a diagram

As is apparent from the equations of the regression in FIGURE 6 (a), 0.95 g P/kg DM was digested when 1 g of P was released from InsPs, provided that no mineral P was added. As the slope does not differ significantly from 1.0 ($P > 0.05$), it might indicate that $InsP\text{-}P_{released}$ was completely digested in a P-deficient state.

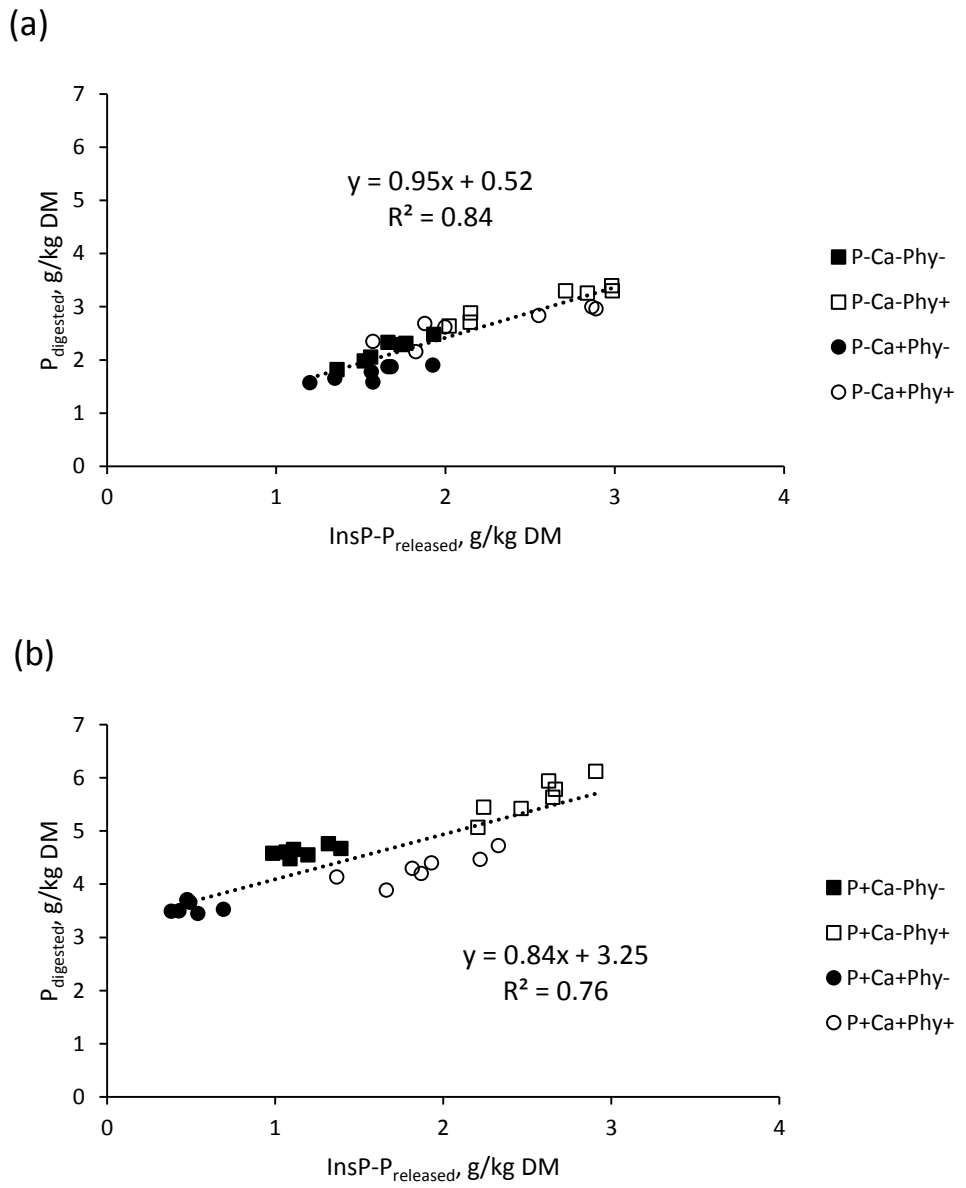

FIGURE 6. Relationship between P released from InsPs and P digested up to the terminal ileum of 27-day-old broiler chickens from the experiment in MANUSCRIPT 2. Diagrams show the treatments without (a) and with (b) P supplementation. Calculation steps are explained in the text. Each symbol represents one of seven replicates per treatment. Ca–, without further Ca supplementation; Ca+ with further Ca supplementation; P–, without P supplementation; P+, with P supplementation; Phy–, without phytase supplementation; Phy+, with phytase supplementation.

However, adding mineral P to the diets led to a lower amount of P digested per g of released P (0.85 g P/kg DM per 1 g of *InsP-P*$_{released}$; FIGURE 6 (b)). The amount of digested P was markedly higher in the treatments with supplemented P, which might have led to a saturation of P absorption with a consequent lower absorption of *InsP-P*$_{released}$.

However, the calculations presented here rely on data from only one experiment and should be extended by data from further experiments where concentrations of $InsP_{1-6}$ were analyzed as well. This calculation was done under the assumption that no InsPs were absorbed or secreted from the epithelial cells into in the ileum lumen. As it is not known whether and to what extent InsPs are absorbed from or secreted into the small intestine, it is not possible to estimate the extent to which these absorption and secretion processes would contribute to the presented calculation. However, it is suitable to show what potential further InsP degradation—not only $InsP_6$—has for P provision of the animal. The graphs also show the difficulty in evaluating different P sources as already stated by Shastak *et al*. (2014). Usually, the digestibility of different P sources is calculated via regression approach. Increasing levels of a P source are supplemented to a P-deficient diet and the P digestibility is determined *in vivo*. The digestibility results are plotted against the inclusion level of the P source and the resulting slope gives the digestibility of the P source. However, from the work of Shastak *et al*. (2014) and the present results, it is obvious that the supplementation of P or P and Ca, e.g. via MCP, decreases the proportion of P released from InsPs that would have been absorbed. This in turn leads to an underestimation of the potential of the investigated P source. This should be kept in mind when phytate-containing basal diets are used in regression approaches or when studies differing in the composition of their basal diets are compared.

3.4 *Myo*-inositol

Broiler studies investigating the effect of an MI supplementation or of MI released by phytase supplementation are scant. TABLE 2 summarizes the studies investigating the effects of dietary P, Ca, phytase, and MI supplementations on the concentration of MI in different segments of the digestive tract or in the blood. TABLE 3 summarizes the studies investigating the effect of phytase or MI supplementation based solely on the performance traits of broiler chickens. Although hints of effects of MI on broiler performance were shown in 1941 by Hegsted *et al*. (1941), with the exception of one study in 1975 (Pearce, 1975), it was not before the 2000s that the publication of studies investigating MI effects continued.

It was questioned in literature whether phytate can be completely dephosphorylated in the digestive tract of the animal. It was suggested that 3- and 6-phytases are not capable of cleaving the P group from Position 2 on the phytate ring (Yu *et al*., 2012). Based on the outcomes of the studies presented in TABLE 2 and TABLE 3, including our own experiments, it now seems that it is possible for broiler chickens to completely dephosphorylate phytate and release MI. Cowieson *et al*. (2015) measured increased MI concentrations in blood after phytase supplementation, wondering whether complete dephosphorylation occurred in the lumen of the digestive tract, the surface of the epithelial cells, or post-absorptive. Based on the results of Walk *et al*. (2014), Beeson *et al*. (2017) and MANUSCRIPTS 2 and 3, it is obvious that the complete dephosphorylation occurs in the lumen of the digestive tract.

Increased MI concentrations due to the supplementation of microbial phytase were found in the crop, proventriculus+gizzard, duodenum+jejunum, ileum, and blood plasma of broiler chickens in the experiments presented in MANUSCRIPTS 2 and 3. This confirms the results of the three studies that investigated dietary effects on MI concentrations in the digestive tract and excreta or the blood of broilers, as summarized in TABLE 2. Higher MI concentrations were found in the gizzard (Beeson *et al*., 2017; Walk *et al*., 2014), the ileum, and the excreta (Beeson *et al*., 2017) of broiler chickens due to phytase supplementation. In the studies by Beeson *et al*. (2017) and Walk *et al*. (2014), higher MI concentrations were analyzed in the treatments with the lower dietary P and Ca concentration compared to treatments supplemented with P and Ca. This was also confirmed in MANUSCRIPT 2. Even in the presence of a high phytase supplementation (> 1,000 FTU/kg), decreasing effects of mineral supplements on the MI concentrations were observed (Beeson *et al*., 2017) with supplementation of P and Ca combined having the greatest effect compared to the treatments with either P or Ca supplementation (MANUSCRIPT 2). Even in the blood, decreased dietary P and Ca or the supplementation of phytase led to higher MI concentrations (Cowieson *et al*., 2015 and MANUSCRIPT 2).

The stomachs are the main action site of the added microbial 6-phytases and the main part of $InsP_6$ disappeared up to the duodenum+jejunum (81% up to duodenum+jejunum vs. 87% up to the terminal ileum in treatment P−Ca−Phy+; MANUSCRIPT 2); thus, it seems likely that most of the MI has been released up to the duodenum+jejunum. The experiment presented in MANUSCRIPT 2 revealed a positive relationship between the concentration of MI in the

stomachs and the concentration of MI in duodenum+jejunum (4.33x – 0.03; R^2 = 0.80; FIGURE 7 (a)).

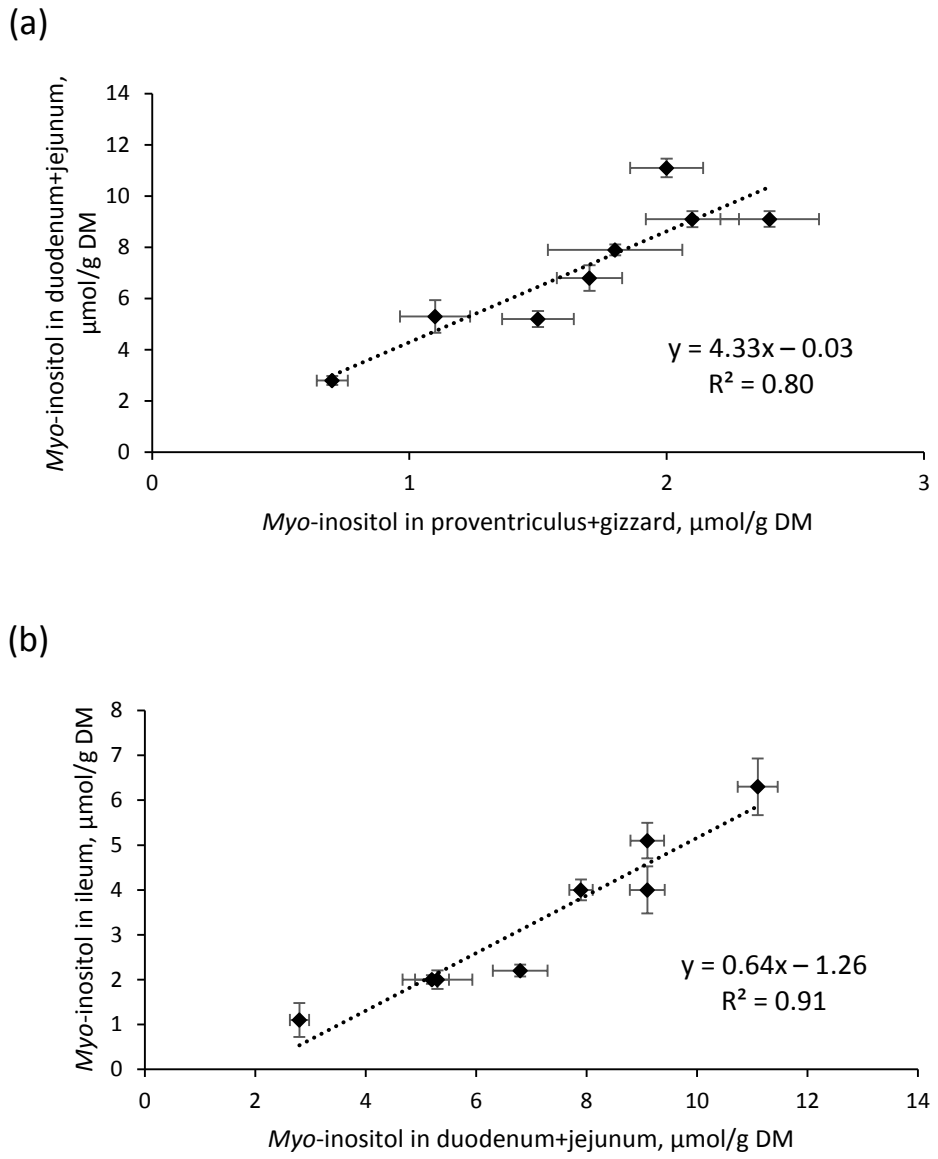

FIGURE 7. Relationships between *myo*-inositol concentrations, corrected with the titanium factor, in the proventriculus+gizzard and duodenum+jejunum (a) and duodenum+jejunum and terminal ileum (b). Each dot represents the mean ± standard error of seven replicates (= pens). Data derived from MANUSCRIPT 2.

To consider that nutrients can concentrate during the passage through the digestive tract, MI concentrations were corrected by using the Ti factor (Ti in feed/Ti in digesta). Otherwise, a direct comparison between concentrations in different digestive tract segments would not be possible. FIGURE 7 (a) shows that more MI was present in the duodenum+jejunum than in the proventriculus+gizzard, possibly due to the high InsP degradation with subsequent MI release. Further, there was a positive relationship between the concentration of MI in the duodenum+jejunum and MI concentration in the terminal ileum (0.64x – 1.26; R^2 = 0.91; FIGURE 7 (b)). The slope reveals 0.64 µmol of MI in the terminal ileum per µmol of MI in the duodenum+jejunum. This indicates that the absorption of MI between duodenum+jejunum and ileum might be higher than the release of MI. It seems that most of the MI was released up to the duodenum+jejunum and that this process slows down up to the terminal ileum. In FIGURE 8, the measured concentration of MI in the terminal ileum is plotted against the concentration of $InsP_6$ (a) and Ins(1,2,3,4,5)P_5 (b) in the terminal ileum, and MI in the plasma (c) (MANUSCRIPT 3). The concentrations in the ileum are based on data from samples pooled per pen. The concentration in the blood plasma is based on data from individual birds. The MI concentration in the ileum increased exponentially with decreasing concentrations of $InsP_6$ (R^2 = 0.77) or Ins(1,2,3,4,5)P_5 (R^2 = 0.72) in the ileum. *Myo*-inositol in blood plasma increased with increasing MI concentrations in the ileum following a power function (R^2 = 0.58), suggesting that the uptake of MI into the blood had a saturation point. Interestingly, the blood MI level was increased by phytase addition in this experiment, but no significant differences were found between the three phytase levels (500, 1,500 and 3,000 FTU/kg feed). Accordingly, the MI concentrations did not significantly differ between treatments with calculated 2,000 or 3,000 FTU phytase/kg feed (Cowieson *et al*., 2015). Contrary to this, the MI concentration in the ileum was increased with increasing phytase dose (MANUSCRIPT 3). As described in MANUSCRIPT 3, the reason for the phytase dose-dependent increase of MI concentrations in the ileum and dose-independent increase of MI concentration in the blood is unknown and needs further investigation. However, it is possible—though highly hypothetical—that metabolic intracellular processes contributed to this outcome.

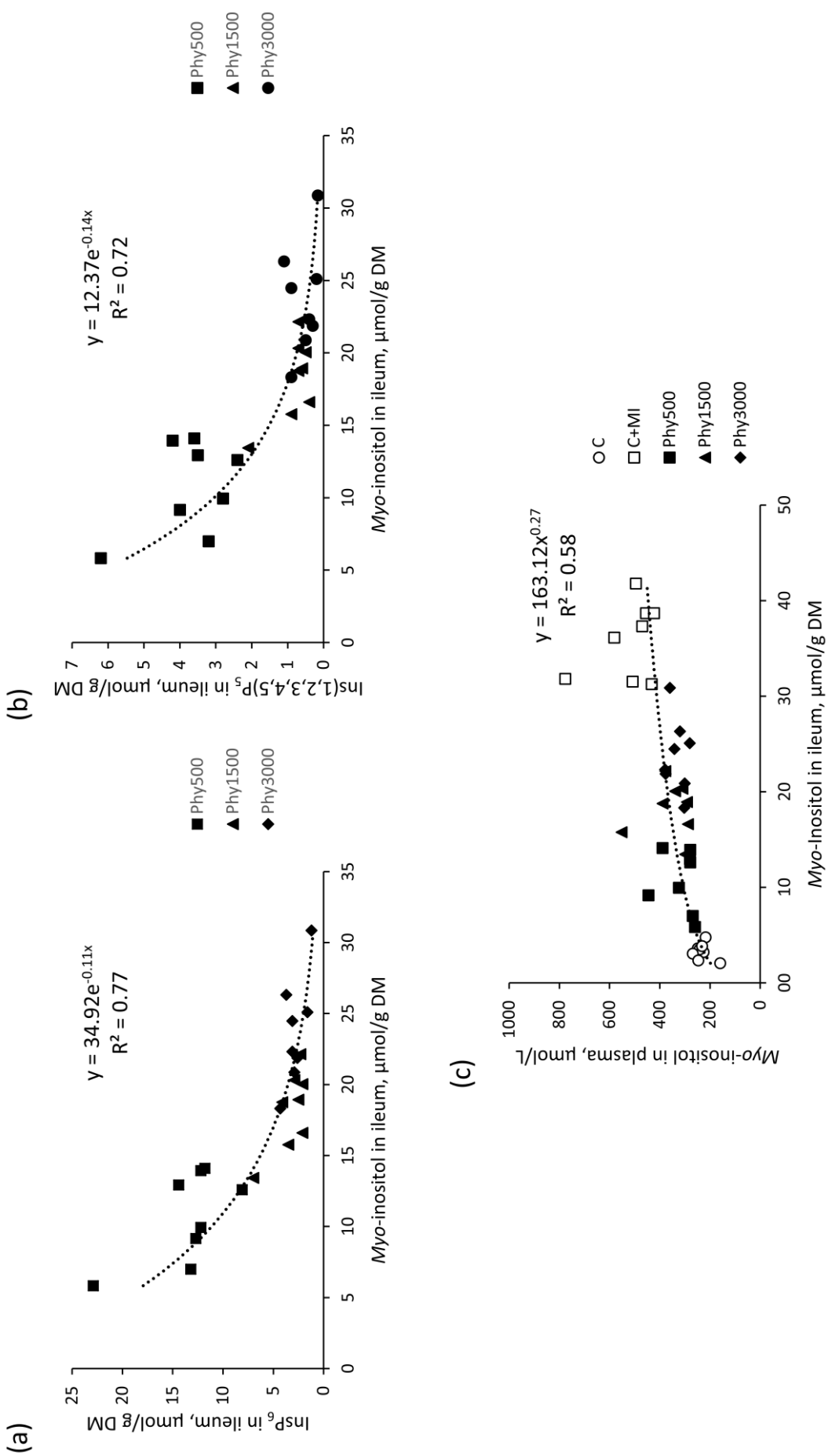

FIGURE 8. Relationships between myo-inositol concentrations in the ileum and concentrations of $InsP_6$ (a), Ins(1,2,3,4,5)P_5 (b) or myo-inositol in blood plasma (c) of broiler chickens. Each symbol represents one replicate (= pen for digesta and individual bird for blood data). Data derived from MANUSCRIPT 3. C, control treatment with adequate P and Ca; C+MI, control treatment with myo-inositol supplementation; Phy500/1500/3000, P and Ca reduced treatment with phytase supplementation.

Further, there might have been a higher absorption efficiency in the anterior parts of the small intestine where the supplemented free MI could have been absorbed immediately, while MI released from phytate during the small intestinal passage would have been absorbed in more posterior segments. In FIGURE 8 (c), the replicates without phytase or MI supplementation hardly vary in their concentrations of MI in blood and ileum, and the range of the results increased with supplementation of MI or phytase. Based on this, one might speculate that some individual birds have a slower MI uptake from blood to organs or tissues or a higher absorption efficiency than other birds at similar MI concentrations in the terminal ileum. This was observed by Lerner and Smagula (1979) in an experiment that entailed incubating a small proportion of the small intestine of six-to-nine-week-old broilers. According to them, MI was transported via diffusion and MI uptake was highly dependent on the individual bird as the influx values ranged between 2 and 113 nmol/g intestine per 10 minutes in more than 100 birds without any effect of age. However, as the ileum data are pooled per pen in the present experiments—thus representing only the mean of several individuals—we do not know the MI concentration in the ileum of the individual sampled for blood. For better differentiation, samples from the digestive tract should also be taken from individual birds. More accurate relationships between MI in different segments of the digestive tract and the blood can then be obtained and individuals with potential lower or higher MI concentrations can be spotted.

Hegsted *et al*. (1941) found an increase in the body weight gain of chickens fed different diets supplemented with 0.1% MI, compared to birds fed non-supplemented diets. Pearce (1975), however, found a decrease in body weight gain of broilers fed diets supplemented with 2.5 g MI/kg feed for eight weeks. A decrease—or a tendency toward a decrease—in the feed conversion ratio (FCR), when calculated for the whole life span, was found in the studies by Cowieson *et al*. (2013) and Żyła *et al*. (2004). During the starter phase, however, Cowieson *et al*. (2013) reported an increase in FCR due to MI supplementation to a diet with adequate dietary P and Ca and no difference between supplemented or non-supplemented P and Ca reduced diets (TABLE 3). In the experiment presented in MANUSCRIPT 3, an increased gain-to-feed ratio (G:F) was obtained in the birds fed the diets supplemented with either MI or phytase when calculated for the grower phase or the whole life span. However, in the starter phase, the increasing effect was only observed for the treatments with added MI or 500 FTU phytase/kg.

One might suggest that the phytase effect on the performance of broilers is derived from a better P and Ca provision due to phytate degradation. However, in the experiment of MANUSCRIPT 3, the amount of absorbed P and Ca and the concentration of P and Ca in blood serum were identical between treatments supplemented with phytase or P, Ca and MI. Moreover, Schmeisser *et al*. (2017) suggested that the phytase effect is not a P and Ca effect per se. In their transcriptomic analysis, broilers receiving a low P and Ca diet with supplemented phytase revealed a significant increase in pathways related to muscle growth compared to birds receiving a diet without phytase or a diet supplemented with P and Ca. They reported a significant upregulation of genes involved in muscle development in the phytase supplemented birds. Two possible mechanisms were hypothesized. First, MI could be rephosphorylated to Ins(1,4,5)P_3, which acts as a second messenger activating signaling pathways linked to muscle growth. Second, MI could act as an insulin-mimetic, by upregulating genes involved in skeletal myogenesis due to activation of insulin-like growth receptors. However, these hypotheses need further investigation.

TABLE 2.Results of studies investigating effects of dietary P and Ca, phytase or myo-inositol supplementation on the concentration of myo-inositol in different segments of the digestive tract or blood plasma. Statistical details are excluded to not impair readability.

Reference	Sampling day(s)	Composition of diets					*Myo*-inositol					
		Main components	P[1]	Ca[1]	MI addition	Phytase activity[1]	Plasma	Crop	Giz(+Prov)	Duo+Jej	Ileum	Excreta
			g/kg			FTU/kg	mmol/L	µmol/g DM				
Beeson *et al*. (2017)[2]	d19-21	C/W/SBM	6.1	10.8	-	-			0.50		9.55	6.72
		C/W/SBM	5.8	10.4	-	744			0.67		12.54	7.83
		C/W/SBM	5.7	10.2	-	1,960			0.78		15.87	8.10
		C/W/SBM	7.5	12.2	-	-			0.44		6.83	2.05
		C/W/SBM	6.7	11.1	-	715			0.61		10.55	4.94
		C/W/SBM	7.1	10.7	-	1,880			0.61		11.49	7.33
Cowieson *et al*. (2015)[3]	d23	C/SBM	4.4	5.6	-	-	0.31					
		C/SBM	4.4	5.6	-	1,049	0.35					
		C/SBM	4.4	5.6	-	2,270	0.37					
		C/SBM	4.4	5.6	-	4,126	0.37					
		C/SBM	7.5	9.9	-	-	0.22					
		C/SBM	7.5	9.9	-	1,122	0.28					
		C/SBM	7.5	9.9	-	2,277	0.32					
		C/SBM	7.5	9.9	-	2,900	0.35					
		C/SBM	8.8	11.8	-	-	0.24					
	d28	C/SBM	4.3	6.0	-	-	0.27					
		C/SBM	4.3	6.0	-	1,287	0.34					
		C/SBM	4.3	6.0	-	2,185	0.35					
		C/SBM	5.7	8.1	-	-	0.25					
Walk *et al*. (2014)	d21	C/SBM	7.2	9.3	-	-			0.91			
		C/SBM	8.2	10.4	-	-			0.88			
		C/SBM	7.2	9.3	-	503			1.15			
		C/SBM	6.0	8.1	-	-			1.21			
		C/SBM	6.0	8.1	-	362			1.57			
		C/SBM	6.0	8.1	-	945			2.12			
		C/SBM	6.0	8.1	-	1,390			2.22			
MANUSCRIPT 2[4]	d25/27	C/SBM	4.5	5.9	-	-	0.24		1.1	12.7	10.1	
		C/SBM	4.5	6.4	-	1,150	0.45		1.3	18.9	16.6	

continues

TABLE 2. continued

Reference	Sampling day(s)	Composition of diets					*Myo*-inositol					
		Main components	P[1]	Ca[1]	MI addition	Phytase activity[1]	Plasma	Crop	Giz(+Prov)	Duo+Jej	Ileum	Excreta
			g/kg			FTU/kg	mmol/L	µmol/g DM				
MANUSCRIPT 2[4]	d25/27	C/SBM	4.3	9.8	-	-	0.18		1.0	9.2	5.7	
		C/SBM	4.4	10.0	-	1,150	0.32		1.4	15.8	11.3	
		C/SBM	7.5	6.2	-	-	0.28		0.7	9.3	5.6	
		C/SBM	7.4	6.2	-	1,160	0.45		1.7	16.8	14.6	
		C/SBM	7.4	10.1	-	-	0.22		0.5	5.5	3.2	
		C/SBM	7.3	9.8	-	1,230	0.32		1.2	12.9	6.5	
MANUSCRIPT 3[4]	d22	W/SBM/C	7.5	9.5	-	-	0.23	1.2			3.3	
		W/SBM/C	8.0	10.1	3.5	-	0.52	12.2			35.9	
		W/SBM/C	6.1	7.9	-	361	0.32	1.3			10.7	
		W/SBM/C	5.9	7.8	-	1,870	0.36	1.4			18.2	
		W/SBM/C	5.9	7.7	-	3,110	0.33	2.4			23.8	

C, corn; Giz, gizzard; Duo, duodenum; Jej, jejunum; MI, *myo*-inositol; Prov, proventriculus; SBM, soybean meal; W, wheat

[1]Analyzed

[2]MI concentrations in this experiment converted from g/kg DM

[3]MI concentrations in this experiment converted from mg/L

[4]Dietary P and Ca concentrations in g/kg DM

TABLE 3. Results of studies investigating the effect of *myo*-inositol supplementation in combination or not with phytase supplementation on the performance traits of broilers. The column "Δ Performance" indicates the difference in body weight gain, feed intake and feed efficiency to the respective treatment without supplementation of phytase or *myo*-inositol. Statistical details are excluded to not impair readability.

Reference	Experimental period	Composition of diets					Δ Performance		
		Main components	P[1]	Ca[1]	MI addition[2]	Phytase activity[1]	Body weight gain[3]	Feed intake[4]	Feed efficiency[5]
			g/kg			FTU/kg	g		g/g
Cowieson *et al*. (2013)	d1-10	W/C/SBM	7.1	8.7	1.5	-	-9	-1	+0.05
		W/C/SBM	6.1	7.0	1.5	-	+2	+8	+0.03
	d11-20	W/C/SBM	6.0	7.3	1.5	-	+4	-3	-0.01
		W/C/SBM	5.1	5.4	1.5	-	-22	+5	+0.06
	d21-42	W/C/SBM	5.8	6.5	1.5	-	-67	-273	-0.08
		W/C/SBM	4.6	5.1	1.5	-	+95	-170	-0.21
	d1-42	W/C/SBM	6.0	7.3	1.5	-	-74	-276	-0.06
		W/C/SBM	5.1	5.4	1.5	-	+75	-157	-0.12
	d1-10	W/C/SBM	7.3	8.5	-	514	+3	+16	+0.04
		W/C/SBM	7.3	8.5	1.5	-	-7	+7	+0.07
		W/C/SBM	7.3	8.5	1.5	514	+6	+12	+0.01
		W/C/SBM	6.2	7.1	-	514	+4	+1	-0.02
		W/C/SBM	6.2	7.1	1.5	-	-11	-7	+0.04
		W/C/SBM	6.2	7.1	1.5	514	+8	-2	-0.06
	d11-20	W/C/SBM	6.3	7.1	-	610	-4	+20	+0.05
		W/C/SBM	6.3	7.1	1.5	-	-4	+25	+0.07
		W/C/SBM	6.3	7.1	1.5	610	+1	-2	0.00
		W/C/SBM	5.0	5.6	-	610	-4	+10	+0.01
		W/C/SBM	5.0	5.6	1.5	-	-17	-14	+0.02
		W/C/SBM	5.0	5.6	1.5	610	+7	+14	-0.01
	d21-42	W/C/SBM	5.9	6.6	-	580	+95	+41	-0.08
		W/C/SBM	5.9	6.6	1.5	-	+134	+110	-0.08
		W/C/SBM	5.9	6.6	1.5	580	+135	+67	-0.10

continues

TABLE 3. continued

Reference	Experimental period	Composition of diets					Δ Performance		
		Main components	P[1]	Ca[1]	MI addition[2]	Phytase activity[1]	Body weight gain[3]	Feed intake[4]	Feed efficiency[5]
			g/kg			FTU/kg	g		g/g
Cowieson *et al*. (2013)	d21-42	W/C/SBM	4.4	5.2	-	580	+56	+43	-0.03
		W/C/SBM	4.4	5.2	1.5	-	+52	+35	-0.03
		W/C/SBM	4.4	5.2	1.5	580	+48	+26	-0.03
	d1-42	W/C/SBM	5.9	6.6	-	500[2]	+94	+78	-0.04
		W/C/SBM	5.9	6.6	1.5	-	+123	+143	-0.03
		W/C/SBM	5.9	6.6	1.5	500[2]	+142	+78	-0.07
		W/C/SBM	4.4	5.2	-	500[2]	+56	+55	-0.02
		W/C/SBM	4.4	5.2	1.5	-	+23	+14	-0.01
		W/C/SBM	4.4	5.2	1.5	500[2]	+64	+39	-0.03
Dam (1944)	start d1	Sucrose/casein/yeast or lard/Cstarch/casein	-	-	1.50%	-	counteracted symptoms of vitamin E deficiency		
Hegsted *et al*. (1941)	4 weeks	Dextrin/casein/cartilage	-	-	0.10%	-	+18		
		Dextrin/casein/cartilage[6]	-	-	0.10%	-	+45		
		Dextrin/casein/cartilage[7]	-	-	0.10%	-	+52		
		Dextrin/casein/cartilage[8]	-	-	0.10%	-	+34		
Pearce (1975)	8 weeks	W/MBM	-	-	2.5	-	-204		
Pirgozliev *et al*. (2007)	d7-17	C/SBM	2.8[2] avP	-	2.5	-	+4.8	+6.4	+0.009
		C/SBM	2.8[2] avP	-	5.0	-	+1.3	+1.4	+0.011
		C/SBM	2.8[2] avP	-	7.0	-	+2.2	+1.8	+0.021
		C/SBM	2.8[2] avP	-	-	500[2]	+3.7	+2.5	+0.05
		C/SBM	2.8[2] avP	-	2.5	500[2]	+5.0	+4.2	+0.046
		C/SBM	2.8[2] avP	-	5.0	500[2]	+4.5	+3.6	+0.046
		C/SBM	2.8[2] avP	-	7.0	500[2]	+2.0	+2.9	+0.010
Żyła *et al*. (2004)	d1-21	Cmeal/SBM	2.7-4.7[2]	6.5-8.0[2]	0.10%	.	+42	+23	-0.1

continues

TABLE 3. continued

Reference	Experimental period	Composition of diets					Δ Performance		
		Main components	P[1]	Ca[1]	MI addition[2]	Phytase activity[1]	Body weight gain[3]	Feed intake[4]	Feed efficiency[5]
			g/kg			FTU/kg	g		g/g
Żyła *et al*. (2013)	d1-21	W or C and SBM	3.6-4.1[2]	5.2-5.5[2]	1.0	-	+58	+52	-0.218
		W or C and SBM	3.6-4.1[2]	5.2-5.5[2]	-	1300[2] AcPU	+82	+87	-0.277
		W or C and SBM	3.6-4.1[2]	5.2-5.5[2]	-	1300[2] AcPU+300[2] FTU	+108	+127	-0.287
MANUSCRIPT 3[9,10]	d1-11	W/SBM/C	7.3	9.7	3.8	-	+1	-1	+0.04
		W/SBM/C	6.7	9.2	-	431	+1	0	+0.03
		W/SBM/C	6.5	8.5	-	2,000	0	-1	+0.02
		W/SBM/C	6.4	8.7	-	3,040	-1	-1	-0.01
	d11-22	W/SBM/C	8.0	10.1	3.5	-	0	-1	+0.01
		W/SBM/C	6.1	7.9	-	361	0	-1	+0.02
		W/SBM/C	5.9	7.8	-	1,870	+1	-2	+0.02
		W/SBM/C	5.9	7.7	-	3,110	0	-2	+0.02
	d1-22	W/SBM/C	8.0	10.1	3.5	-	0	-1	+0.02
		W/SBM/C	6.1	7.9	-	361	0	-1	+0.02
		W/SBM/C	5.9	7.8	-	1,870	0	-2	+0.02
		W/SBM/C	5.9	7.7	-	3,110	-1	-2	+0.02

C, corn; Cmeal, corn meal; MBM, meat and bone meal; MI, myo-inositol; SBM, soybean meal; W, wheat

[1]Analyzed, unless otherwise indicated

[2]Calculated

[3]Cowieson, Zyla, Zyla: body weight gain (g) during period; Hegstedt, Pearce: body weight (g) at end of the experiment; Pirgozliev, MANUSCRIPT 3: average daily gain (g)

[4]Cowieson, Zyla, Zyla: feed intake (g) during period; Pirgozliev: average daily feed intake (g DM); MANUSCRIPT 3: average daily feed intake (g)

[5]Cowieson, Zyla, Zyla: feed-to-gain ratio (g/g); Pirgozliev, MANUSCRIPT 3: gain-to-feed ratio (g/g)

[6]Without kidney residue and with 10% molasses

[7]With liver extract

[8]With alcohol soluble yeast eluate

[9]Dietary P and Ca concentrations in g/kg DM

[10]Performance differences were calculated to the control treatment with adequate dietary P and Ca

3.5 Effects of dietary P and Ca and phytase supplementation on prececal amino acid digestibility

Results from existing literature investigating the effects of phytase supplementation on prececal AA digestibility in broiler chickens are summarized in ANNEX 1. When comparing the results from literature, it becomes obvious that there is no clear pattern of the effectiveness of phytase supplementations on AA digestibility. Several studies reported an increase of prececal AA digestibility (for example Amerah *et al*., 2014; Ravindran *et al*., 1999; Rutherfurd *et al*., 2002), whereas others report no effect (for example Peter and Baker, 2001; Rodehutscord *et al*., 2004; Sebastian *et al*., 1997) or even a negative effect (for example Liu *et al*., 2016) of phytase on the prececal digestibility of at least some AAs. The outcomes of the studies vary to a great extent, as do the conditions of the respective studies. The studies differ in terms of strain and age of the broiler chickens and the duration of the experimental feeding. Further, diets vary in P, PP, Ca, protein, and phytase source and concentration. The indigestible marker used for calculating the prececal digestibility of nutrients can vary and is known to affect the outcome of AA-digestibility experiments, as discussed later. Variation between studies may also be caused by the chosen sacrificing and sampling procedures and the methods used in the laboratories to measure AAs, the marker, and other nutrients. Some possible influencing factors have already been reviewed by Kluth and Rodehutscord (2009). In the broiler experiments of the present work, phytase effects on the prececal AA digestibility were found. In MANUSCRIPT 2, the supplementation of phytase increased the AA digestibility between two and six percentage points, independent of the dietary P or Ca concentration. In the second experiment, presented in MANUSCRIPT 3, a phytase effect was found only with the inclusion levels 1,500 or 3,000 FTU/kg, but not for all AAs. Based on these findings, one might suggest that the effect is dose-dependent and does not apply to a phytase supplementation per se. The results and even the conditions and set-ups of the experiments presented in literature vary a lot, but it is difficult to find a reason for this variation. As the AA ring test (Ravindran *et al*., 2017) revealed that the AA digestibility is basically similar over several research stations if a standard protocol was applied, another ring test with and without phytase supplementation would help to determine whether phytase effects still vary when working with the proposed protocol. If this is the case, the varying results on $InsP_6$ degradation and P digestibility found in the P ring test (Rodehutscord *et al*., 2017) might also contribute to

the phytase efficacy on AA digestibility; the reason for the variation in phytase effects on AA digestibility might be the same as for the variations in P utilization. The following paragraph gives an overview of the possible interactions of phytate and AAs and factors that might affect them. For deeper insights, the reader is referred to the reviews by Morales *et al*. (2016), Selle *et al*. (2000, 2006, and 2012), and Selle and Ravindran (2007).

3.5.1 Interactions between phytate, phytase and amino acids

Binary phytate-amino acid complexes

$InsP_6$-protein complexes are mainly formed by interactions between the anionic phosphate groups of $InsP_6$ and the cationic AA residues of proteins at pH values below the isoelectric point of proteins (Morales *et al*., 2016; Selle *et al*., 2000). Kies *et al*. (2006), in their work on the interactions between protein, phytate, and phytase *in vitro*, concluded that protein-phytate complexes are mainly formed at low pH (= 2). There was no formation of binary complexes at a pH > 4. Also, the addition of phytase prevented the formation of complexes or helped dissolving them. In the presence of phytase, pepsin released the protein from such complexes to a larger extent than without phytase. Thus, it is obvious from this study that the pH is an important factor in the formation of binary complexes. This applies mainly to the pH of the stomach(s) of animals, which can range between 1.6 and 5.7 in broilers, as reviewed by Svihus (2011). In addition, this means that these complexes might be dissolved when the pH (along digestive tract) increases. Apart from pH, other factors can influence the formation of binary complexes, such as Ca^{2+} (Morales *et al*., 2016); these are discussed later. Binary complexes may lead to a reduced proteolysis by pepsin, as confirmed in several studies reviewed by Selle *et al*. (2000; 2012). Selle *et al*. (2000) also concluded that the addition of phytase may possibly prevent such complexes rather than dissolve the existing complexes where proteins surround the substrate $InsP_6$.

The formation of phytate-protein complexes at low pH means that phytases with an optimum at low pH (for example *E. coli*-derived phytases as utilized in MANUSCRIPTS 2 and 3) should be more likely to diminish such complexes than phytases with higher pH optimum (for example *A. niger*-derived phytases). Depending on the feedstuff and Ca concentration and source, for example, the pH may be altered in the anterior segments of the digestive tract (see GENERAL INTRODUCTION). This might lead to a shift in the phytase efficacy. The choice of the phytase and

the diet composition therefore seems to be crucial for binary complexes and might also contribute to the differences in the results found in literature (ANNEX 1). As the experimental setup varies widely between experiments, for example in P, phytate and Ca concentrations, age and strain of birds and other factors, a direct comparison between different phytase sources is hardly possible. However, tendencies toward the effects of different phytase sources might be determined by a meta-analysis.

Ternary phytate-amino acid complexes

Posterior to the stomachs, the pH rises along the digestive tract and binary $InsP_6$-protein complexes might be dissolved. In the small intestine, most proteins reach their isoelectric point. $InsP_6$ and proteins are now negatively charged and can build complexes with cations. As Ca^{2+} is the most prevailing cation in the digestive tract, $InsP_6$-Ca^{2+}-protein complexes are common. $InsP_6$ can also form binary complexes with cations. However, the ratio between $InsP_6$-cation complexes and $InsP_6$-cation-protein complexes in the small intestine is not known (Morales *et al*., 2011). The formation of these ternary complexes has consequences for not only protein or AA digestibility but also the digestibility of the cations that are bound to the complexes. Phytases are suggested to degrade $InsP_6$ before such complexes can be formed, thereby reducing potential ternary complexes. However, Morales *et al*. (2011), in an *in vitro* experiment simulating gastric (pH 2.5) or intestinal (pH 8.5) digestion in fish, found that the addition of $InsP_6$ to casein as substrate led to a reduced release of AAs in gastric phase but not in the intestinal phase. Also, the pre-digestion of soybean meal as substrate with phytase led to increased AA release at pH 2.5 but not at pH 8.5. This leads to the suggestion that ternary complexes do not play a major role in diminishing protein digestibility. Moreover, this implies again that the phytase effect on AA digestibility would be more pronounced using phytases with an optimum at pH values prevailing in the anterior segments of the digestive tract, degrading phytate at an early stage.

Another proposed phytate-protein interaction is phytate acting as a Hofmeister anion (Kidd *et al*., 2016; Morales *et al*., 2016; Selle *et al*., 2012). According to this hypothesis, the negatively charged phytate is a kosmotrope that can change the thermodynamics of water (Cowieson and Cowieson, 2011) resulting in a stabilization of proteins. For further information about the ability of cations and anions to (de)stabilize proteins the reader is referred to Baldwin (1996).

Phytate could therefore reduce the solubility of proteins without direct binding. Cowieson and Cowieson (2011) proposed that the interaction between phytate and proteins is mainly an indirect one and the direct binary or ternary binding is not the main mechanism. Further studies should investigate the relevance of these interactions.

Influence of feed ingredients

In the study by Ravindran *et al*. (1999), negative correlations were found between the inherent protein (and mean AA) digestibility and the phytase response in protein (and mean AA) digestibility ($r = -0.42$, $P < 0.05$ for protein and $r = -0.51$, $P < 0.01$ for AA). This led to the conclusion that feedstuff with poor AA digestibility responds better to phytase supplementation. Ravindran *et al*. (1999) also reported negative correlations ($r = -0.81$, $P < 0.001$) between the dietary $InsP_6$ concentration and the inherent protein or mean AA digestibility. This means that the phytate concentration in feedstuff has a substantial effect on the digestibility of AAs and protein. Further, in the studies by Ravindran *et al*. (1999) and Rutherfurd *et al*. (2002), a higher increase in AA digestibility due to phytase supplementation was reported in diets based on wheat compared to diets based on corn (see ANNEX 1). As the correlation between dietary $InsP_6$ concentration and phytase response in protein and AA digestibility was not significant, the conclusion was that the differences in phytase response between feedstuffs was not based mainly on the phytate content, as described above, but more on the structural and chemical properties of phytate and protein in the feedstuff. Confirmative, Morales *et al*. (2013) found that the efficacy of phytase to enhance protein solubilization was dependent on the feedstuff used. There was a greater effect of phytase on protein fractions dominating in peas or broad beans (convicilin, vicilin and legumin) than on protein fractions found in soybeans (glycinin and β-conglycinin), and only minor phytase effects were found on lupins (conglutin), wheat (gliadins and glutenins), or canola meal (oleosin and napin), evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Unfortunately, corn was not part of the study. It was suggested that the phytase effects were due to the AA composition of the storage proteins, as vicilin and legumin are known to contain a high proportion of basic AAs, while gliadins and glutenins only have a small proportion. A higher proportion of basic AAs could lead to an increased formation of binary protein-phytate complexes at low pH, as described earlier.

The phytase response on prececal AA digestibility was higher in the experiment of MANUSCRIPT 2, having a lower inherent prececal AA digestibility than in the experiment of MANUSCRIPT 3, where the inherent AA digestibility was already on a high level. This is supported by the aforementioned argument that feedstuff with low inherent AA digestibility responds better to phytase. However, the results of MANUSCRIPTS 2 and 3 contradict the second argument that wheat responds better to phytase than corn. As these experiments differed in not only their main ingredients but a lot of other factors as well, a direct comparison should be done with caution.

Effect of phytate on endogenous losses

Phytate is known to increase specific endogenous losses. Cowieson *et al*. (2004) reported that the addition of phytate to glucose, which was given to precision-fed broilers, led to higher excretions of endogenous nitrogen, AAs, Na, and sialic acid. Additional phytase supplementation alleviated this effect. Sialic acid—in this context—is an indicator of mucin production. Liu and Ru (2010) reported an increase in endogenous AAs due to phytate supplementation in broiler chickens using purified diets. This was also found by Cowieson *et al*. (2004) and Onyango *et al*. (2009), whereby Onyango *et al*. (2009) found higher endogenous losses with the use of Mg-K-phytate than with free $InsP_6$, suggesting a dependency on the phytate source. Under the assumption that $InsP_6$-protein complexes are less susceptible to pepsin digestion, these complexes could trigger higher gastric secretions of pepsin and HCl as compensation (Selle *et al*., 2012). To buffer this, a higher secretion of $NaHCO_3$ and mucin follows. A higher production of $NaHCO_3$ from free Na may have detrimental effects on the Na-K-ATPase activity in the small intestine and therefore on the transport of peptides (Cowieson *et al*., 2009). Liu *et al*. (2008) demonstrated that phytate decreased the concentration of Na-K-ATPase in the jejunum of broiler chickens. However, P and K in the digestive tract can also be influenced by phytate or phytase and therefore influence the transport of peptides, for instance (Liu *et al*., 2008). Basal endogenous losses may also contribute to AA digestibility. However, they are dependent mostly on DM intake and not on the properties of the feedstuff. Therefore, basal endogenous losses are discussed later.

Influence of dietary P and Ca

Martinez-Amezcua *et al*. (2006) reported an increased prececal digestibility for some AAs when 1,000 FTU phytase/kg were applied and for all AAs when 10,000 FTU/kg were supplemented. However, the increase in AA digestibility was also achieved or even exceeded when KH_2PO_4 instead of phytase was supplemented to the P-deficient diet. They suggested that the observed phytase effect was not a nutrient (AA)-releasing effect per se but an effect caused by the provision of P that is needed for the Na-K-ATPase pump, for example, to transport the AAs out of the digestive tract. This effect could also be seen in the work of Dilger *et al*. (2004). Centeno *et al*. (2007) determined a higher digestibility of most AA by adding 1.3 g avP/kg or 500 FTU phytase/kg to a diet with 1.4 g avP/kg. Increasing dietary avP to 3.7 g/kg, however, did not have further effects. Onyango *et al*. (2005) observed no phytase effect on the prececal digestibility of AAs and the highest digestibility values were found in the adequate P diet or the low P diet with added 0.75 g P_i/kg. Pieniazek *et al*. (2017) observed an increase in prececal digestibility of all AAs in 21-day-old broilers with the supplementation of P and Ca or 250 FTU phytase/kg and an increased digestibility of some AAs with 500 or 2,000 FTU/kg. In 42-day-old broilers, the increase by phytase was only achieved with 2,000 FTU/kg or P and Ca supplementation. This P effect is not consistent throughout studies—for example, no significant effect of P supplementation was found in the experiment of MANUSCRIPT 2—and needs further investigation. Except for the study by Martinez-Amezcua *et al*. (2006), feed intake was increased due to P or phytase supplementation in all cited studies where P supplementation led to an increased prececal AA digestibility. How this could have influenced the outcome of the studies is discussed later.

Shafey and McDonald (1991) reported a reduced prececal AA digestibility due to excess dietary Ca and avP. They found that Ca increased the microbial population in the digestive tract. Based on the studies by Coates *et al*. (1955) and Lev *et al*. (1957), Shafey and McDonald (1991) concluded that an increased microbial population might have led to an increase in the intestinal wall thickness which might have caused a decreased absorption capacity of AAs. This was supported by the finding that the addition of antimicrobials reversed the Ca effect on AA digestibility. Morales *et al*. (2016) and Selle *et al*. (2009a) suggested that Ca^{2+}—having a high acid-binding capacity—can compete with proteins for the active sites of the negatively

charged $InsP_6$. Thus, it may dissociate binary $InsP_6$-protein complexes in the anterior digestive tract at low pH. Moreover, high dietary Ca may lead to an increased pH in the anterior digestive tract segments, which could diminish the formation of binary $InsP_6$-protein complexes (Selle *et al*., 2009a). It was also reported by Selle *et al*. (2009a) that Ca has the capacity to directly bind proteins thus decreasing their solubility, whereby the pH at which these interactions happened varies widely between studies. As mentioned earlier, Ca also has the capacity to form ternary complexes with $InsP_6$ and proteins or AA at pH prevailing in the small intestine.

Influence of feed intake

Butts *et al*. (1993) found increased endogenous prececal AA and N excretion with increasing feed DM intake in pigs weighing 50 kg. However, they could not determine whether this outcome was due to a specific feed ingredient or the higher feed DM and which kind of endogenous losses was influenced by this factor. They proposed several possible effects of a higher feed intake on endogenous losses: Higher dietary protein intake may lead to increased protein secretion of the pancreas. Higher dietary fat intake may lead to increased lipase secretion of the pancreas. Higher dietary starch intake may lead to increased amylase secretion of the pancreas and increased brush border sugar hydrolases. Higher dietary fiber intake may lead to increased gastric, pancreatic and intestinal secretions. Moter and Stein (2004), in an experiment with growing pigs, found higher endogenous losses of AAs with higher feed intake when calculated as amount per day. However, when calculated as a proportion of DM intake, endogenous losses decreased with increasing feed intake, which was also reported by Stein *et al*. (1999). Moter and Stein (2004) found that the apparent prececal digestibility increased for most AAs when feed intake increased. They suggested that the proportion of the endogenous AAs in total AAs (endogenous and undigested AAs) in the ileum was higher with lower feed intake, resulting in a lower calculated apparent digestibility. However, this is only observable at a very low feed intake level. At higher intake, the contribution of endogenous AA losses to total AAs decreases, resulting in a lower effect of increased feed intake at high levels. Moter and Stein (2004) also found a decreased standardized prececal AA digestibility with increasing feed intake. As the endogenous losses are excluded in the calculation, the decrease in digestibility must have been caused by the increased intake of AAs. Siegert *et al*. (submitted), investigated the effect of different feed

intake levels on the prececal AA digestibility of two test ingredients (rapeseed cake and toasted soybeans) using two basal diets with the regression approach according to Rodehutscord *et al*. (2004). They found differences in feed intake between the diets based on the basal diet that was deficient in essential AAs and no differences between diets based on the basal diet with adequate AA concentrations. The differences found for diets based on the basal diet deficient in essential AAs might have led to different proportions of basal endogenous losses, resulting in an inaccurate estimate of the AA digestibility of the two test ingredients.

In some studies, the supplementation of P or phytase resulted in an increased AA digestibility and—in most studies—also to an increased feed intake, as described earlier. An increased feed intake could have two consequences. It might lead to a decreased proportion of the basal endogenous losses of total AAs in the digesta and thus an increased prececal AA digestibility. This might be the case until a plateau is reached where the percentage share of basal endogenous losses of the total AAs in digesta becomes negligible. If the feed intake level is then further increased, this may lead to a different effect. A higher feed intake may lead to a faster passage rate, resulting in a lower nutrient utilization (Svihus, 2010), and to a higher filling of the intestinal tract, which might reduce the accessibility of the nutrients for enzymes. Further, a higher feed intake means a higher amount of AAs, which might lead to an increased secretion of digestive enzymes (Butts *et al*., 1993) and thus higher specific endogenous losses. Therefore, a further increase in a high level of feed intake might lead to an underestimation of the increasing effect of phytase or P. However, it is hardly possible to determine the level of feed intake in which the basal endogenous losses are the main factor to consider and the feed intake level where the digestibility of nutrients decreases with increasing feed intake. A meta-analysis taking the feed intake into account could help shedding some light on this issue.

Taking the observations of these studies into account, it cannot be ruled out that the significantly lower feed intake in the treatment P−Ca+Phy− in the broiler experiment described in MANUSCRIPT 2 influenced the outcome of the AA digestibility. Therefore, studies investigating the prececal AA digestibility in dependence of phytase supplementation should consider a standardized feed intake for all treatments. To study the phytase effects on specific feed

ingredients, a regression approach should be used (Rodehutscord *et al*., 2004), which includes the endogenous losses in the resulting slope and therefore needs no further correction.

Influence of the indigestible marker

As reviewed by Selle *et al*. (2006), Selle and Ravindran (2007), and Cowieson and Bedford (2009), the choice of the indigestible marker can influence the outcome of studies investigating phytase effects on the AA digestibility in poultry. It was reported that the effect of phytase on prececal AA digestibility, calculated as percentage increase, was lowest with the use of chromic oxide (Cr_2O_3), higher with the use of titanium oxide (TiO_2), and highest with acid insoluble ash. In contrast, the prececal AA digestibility—particularly for the basal diet—was highest with the use of Cr_2O_3, suggesting an overestimation of the AA digestibility in the basal diet and thus a lower improvement by phytase. Moreover, it was reported that Cr_2O_3 is not evenly distributed in the digesta and the analysis of Cr_2O_3 suffers from low recovery and issues due to the presence of P in digesta. Olukosi *et al*. (2012), however, reported contradictory results. In their experiment, Cr_2O_3 and TiO_2 were included simultaneously in the experimental diets for 42-day-old broilers and after 7 days, prececal AA digestibility was determined with either of the two markers (ANNEX 1). They found higher AA digestibility values with TiO_2, independent of phytase addition, and no consistent phytase effect on the AA digestibility. They traced the lack of phytase effect back to the high level of AA digestibility even without phytase supplementation (as discussed earlier). The marker concentration varying between 0.3 and 0.5% had no influence on digestibility. Therefore, it might be possible that the choice of the indigestible marker may contribute to the magnitude of the phytase effect on AA digestibility and thus to the varying results found in the literature.

3.5.2 Relationship between amino acid digestibility and inositol phosphate isomers

Some recent studies suggested a direct decreasing effect of lower InsP esters—namely $InsP_3$ and $InsP_4$—on the prececal digestibility of AA (Bedford and Walk, 2016). Based on correlations between $InsP_4$ or $InsP_3$ and the digestibility of energy, nitrogen, and several minerals, they concluded that $InsP_3$ and $InsP_4$ may also have negative effects on the digestibility of AAs, just like $InsP_6$. The results from the experiment presented in MANUSCRIPT 3 and investigated in the context of MANUSCRIPT 2 led to a different conclusion. In the experiment described in MANUSCRIPT 2, positive relationships were found between the concentration of Ins(1,2,5,6)P_4

or Ins(1,2,3,4,5)P_5 and the prececal digestibility of most AAs. These outcomes do not rule out a direct effect of lower InsP esters and their isomers on the digestibility of AAs or proteins. However, as explained in MANUSCRIPT 3, it is more likely that the relationship between InsP esters and AA digestibility is not a causal one. It is more likely that it is a coincidental relationship between the phytate-degrading and AA-digestibility-enhancing effect of phytase. However, the correlation coefficients of the AAs that are suggested to be associated with phytate degradation (Cowieson and Bedford, 2009), were not higher than for other AAs. The work of Yu *et al*. (2012) shows that the binding capacity of InsPs on soy protein and β-casein *in vitro* decreased substantially from $InsP_6$ to $InsP_4$, whereby the binding capacity of $InsP_4$ and lower InsPs was negligibly low. They also found that Ins(1,2,3,4,5)P_5 produced by 6-phytases had a lower protein binding capacity than Ins(1,2,4,5,6)P_5 produced by 3-phytases. $InsP_3$ and $InsP_4$ were still able to inhibit pepsin activity, but to a lesser extent than $InsP_5$ or $InsP_6$. This was investigated in a medium at pH 1.6–4.5, whereby no aggregation occurred below pH 2.0. Morales *et al*. (2011) found that the release of AAs from casein *in vitro* at pH 2.5 was decreased when $InsP_6$ was added. At pH 8.5, however, there was no effect of $InsP_6$ on AA release, suggesting that the ternary phytate-cation-protein complexes in the small intestine do not play a major role in the provision of AAs for the bird. Consequently, lower InsP esters with an even lower binding capacity would contribute even less to a diminished AA digestibility.

Based on the plethora of possible influencing factors on the AA digestibility in poultry —especially on the phytase effect—a general statement on phytase affecting AA digestibility cannot be made. This field of research needs further systematic approaches.

3.6 Perspectives for future research

A great variability exists regarding $InsP_6$ disappearance and effects of the dietary composition on it across the studies. With the ring test of Rodehutscord *et al*. (2017), it is apparent that results may vary despite adhering to an experimental protocol. Thus, more research is needed to investigate the reasons for this variation and—if necessary—to adapt the protocol. Until this happens, direct comparisons between studies have to be done with caution, because effects of P, Ca, and phytase found in one study, for example, would not necessarily be found in another study.

This point leads to the necessity of interdisciplinary research. The measured $InsP_6$ disappearance is the result of an interplay of the animal's genetic, microbiome, nutrition, and physiology. However, studies mostly focus on a single discipline, although the information value could be greatly increased by engaging several research groups in one experiment. It is therefore highly recommended to work together in interdisciplinary experiments aiming to contribute to fill the gap of knowledge of these complex processes.

It is still not known how much the endogenous epithelial and microbial phytases and phosphatases respectively contribute to the degradation of $InsP_6$ and lower InsP isomers. This could be investigated via an experiment with gnotobiotic birds. The contribution of the endogenous epithelial phytase could be studied with the use of germfree birds. Based on the outcomes, gnotobiotic birds with a targeted colonization of single microorganisms or a defined microbial population could help to define the relevance of these two phytase and phosphatase sources for the bird, with the aim of finding possibilities to influence the bird's own potential to utilize PP.

There is a great variation in the results of experiments investigating the phytase effect on AA digestibility in poultry. Thus, it should begin with a meta-analysis evaluating all available data in literature with the aim to find factors and especially interactions influencing the phytase effect on AA digestibility, that go beyond the results of the existing meta-analysis by Cowieson *et al*. (2017). If potential factors or interactions are found throughout all experiments, a systematic investigation of these factors should be undertaken. These experiments should be carried out with a standardized feed intake to exclude variations in the results due to varying basal endogenous losses. The regression approach suggested by Rodehutscord *et al*. (2004) can be used to study the phytase effects on specific feed ingredients without the need for a correction of the basal endogenous losses.

There is now some evidence that MI can be generated during the passage of the digestive tract, especially when a high dose of phytase is supplemented. Moreover, MI was found to increase the feed efficiency of broilers in some experiments, including the present one. Now, the metabolic mode of action of MI should be investigated and further studies should be conducted to confirm or reject the outcomes and prove the benefit derived from phytases apart from the P-releasing effect.

3.7 Conclusion

It can be concluded that the established *in vitro* assay is a suitable tool to investigate effects on feed enzymes or differences between feed enzymes in a feed matrix under standardized conditions without the interference of endogenous phytases or depending on animal-specific variations. It might, therefore, help to reduce the number of animals used in *in vivo* experiments. Due to the complexity of the processes in living animals, it is not possible to replace *in vivo* experiments completely.

Based on the outcome of the present *in vitro* and *in vivo* experiments, the combined supplementation of P and Ca—rather than supplementation of P or Ca solely—seems to be crucial for InsP degradation. This leads to the suggestion that P and Ca should be supplemented as little as possible to maximize the utilization of $InsP_6$-P without risking a deficiency. As the results regarding the effects of P and Ca on $InsP_6$ degradation are not consistent across studies, further research is needed. It would be beneficial to first investigate the reasons for the great variability observed in P-digestibility studies, with subsequent adaption of the existing experimental protocol. Phytase supplementation increased the concentration of MI in the digestive tract and blood, suggesting that phytate can be completely dephosphorylated in the digestive tract. The digestibility of AAs was found to be increased by phytase addition, whereas dietary P and Ca had no significant effect. Due to the great variation between studies regarding phytase effect on AA digestibility, a comprehensive meta-analysis should be carried out to investigate single and interactive effects. Based on this, systematic studies should be executed to shed light on this topic.

Moreover, there is some evidence that MI can increase the feed efficiency of broiler chickens without affecting the metabolism of InsPs or AAs. As literature is still scarce and results are not consistent across studies, further experiments should be conducted.

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4 INCLUDED MANUSCRIPTS

4.1 MANUSCRIPT 1

Modification and application of an *in vitro* assay to examine inositol phosphate degradation in the digestive tract of poultry

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Abstract

An *in vitro* assay was modified to study the disappearance of *myo*-inositol hexakisphosphate ($InsP_6$) and the formation of lower inositol phosphate (InsP) isomers in the poultry digestive tract. Three experiments investigated the influence of diets with different ingredients and additives. The assay simulated the conditions (e.g. temperature, pH, proteolytic enzymes, water content, and retention time) of the crop, stomach, and small intestine, using the poultry diet as a matrix. The extraction and analysis of InsP isomers were immediately conducted. The assay produced highly reproducible results with coefficients of variation ≤10% for an InsP isomer concentration ≥0.4 μmol g^{-1} DM (n = 3). It was sensitive to the factors that varied in the three experiments. The described assay is a suitable tool to screen feed enzymes and to investigate the effects of supplements in the absence of endogenous phytases. The ease of handling and high reproducibility of the assay indicated that the assay is a rapid and feasible method that can be used to examine the degradation pathway of $InsP_6$ in feed under gastrointestinal conditions.

4.2 MANUSCRIPT 2

Interactive effects of phosphorus, calcium, and phytase supplements on products of phytate degradation in the digestive tract of broiler chickens

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Abstract

This study aimed to distinguish between the single and interactive effects of phosphorus (P), calcium (Ca), and phytase on products of phytate degradation, including the disappearance of *myo*-inositol (MI), P, Ca, and amino acids (AA) in different segments of the digestive tract in broiler chickens. Furthermore, all dephosphorylation steps from myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) ($InsP_6$) to MI were investigated in the digesta of the terminal ileum. Unsexed Ross 308 broiler chickens were allocated to 56 pens with 19 birds per pen, and assigned to one of 8 dietary treatments. The dietary treatments included diets without (P−, 4.1 g/kg DM) or with (P+, 6.9 g/kg DM) monosodium phosphate supplementation, without (Ca−, 6.2 g/kg DM) or with (Ca+, 10.3 g/kg DM) additional fine limestone supplementation, and without or with 1,500 FTU phytase/kg feed in a factorial design. When phytase was added, Ca or P supplementation had no effect on $InsP_6$ disappearance in the crop. When no phytase was added, $InsP_6$ disappearance up to the terminal ileum (P−Ca− 56%) was decreased in P+Ca− (40%), and even more so in P+Ca+ (21%). Adding phytase removed all effects of P and Ca (77 to 87%); however, P+Ca+ increased the concentrations of lower InsP esters and reduced the concentration of free MI in the ileum, even in the presence of phytase. These results indicate that mineral supplements, especially P and Ca combined, reduce the efficacy of endogenous microbial or epithelial phosphatases. Supplementation with phytase increased, while supplementation with Ca decreased the concentration of MI in all segments of the digestive tract and in blood plasma, demonstrating the ability of broilers to fully degrade phytate and absorb released MI. While AA disappearance was not affected by P or Ca, or an interaction among P, Ca, and phytase, it increased with the addition of phytase by 2 to 6%. This demonstrates the potential of the phytase used to increase AA digestibility, likely independent of P and Ca supply.

4.3 MANUSCRIPT 3

Influence of phytase or *myo*-inositol supplements on performance and phytate degradation products in the crop, ileum, and blood of broiler chickens

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Abstract

The objective of this study was to investigate the effects of supplementation with free *myo*-inositol (MI) or graded levels of phytase on the degradation of inositol phosphates (InsP), concentrations of MI in the digestive tract and blood, bone mineralization, and prececal digestibility of amino acids (AA). Ross 308 broiler hatchlings were allocated to 40 pens with 11 birds each and assigned to one of 5 treatments. Until d 11, the birds were fed a starter diet and from d 11 to d 22, they were fed a grower diet. All diets were based on wheat, soybean meal, and corn. Birds were fed a control diet without (C) or with MI supplementation (C+MI), or one of 3 experimental diets that differed in phytase level (modified E. coli-derived 6-phytase; Phy500, Phy1500, or Phy3000 FTU/kg). Diets C and C+MI were calculated to contain adequate levels of all nutrients. Diets Phy500, Phy1500, and Phy3000 had P and Ca levels adapted to the recommendations of the phytase supplier for a phytase level of 500 FTU/kg. The gain:feed ratio (G:F) was increased by 0.02 g/g by MI or phytase in the starter+grower phase. Prececal P and Ca digestibility, P and Ca concentration in blood serum, and tibia ash weight did not differ among treatments ($P > 0.05$). MI supplementation led to the highest MI concentration in the crop, ileum, and blood plasma across treatments. Phytase supplementation increased MI concentrations in the crop and ileum digesta in a dose-dependent manner and in plasma without any dose effect ($P > 0.05$). Prececal digestibility of some AA was increased by phytase. These outcomes suggest that MI might have been a relevant cause for the increase in G:F. Therefore, it is probable that the release of MI after complete dephosphorylation of phytate is one of the beneficial effects of phytase, in conjunction with the release of P and improvement in digestibility of other nutrients. Concurrently, MI seems to have no diminishing effects on InsP degradation.

5 SUMMARY

Phosphorus (P) is an important element in poultry nutrition, which must be adequately supplied in the diet. For non-ruminant animals, however, it is only partially available from plant seeds, where P is predominantly bound as phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); $InsP_6$) and its salts, called phytate. Phytate-P (PP) can be utilized after the stepwise cleavage of the P groups from the phytate molecule catalyzed by phytases (*myo*-inositol hexaphosphate phosphohydrolases) and other phosphatases. After the theoretical complete dephosphorylation of $InsP_6$, six P groups and *myo*-inositol (MI) are potentially available for absorption. *Myo*-inositol is suggested to have several biological functions, for example in cell survival and growth, lipid metabolism, and insulin sensitivity. Recent studies assume an effect of MI on the growth performance of broiler chickens when it is added in its free form to the diet or released as a result of $InsP_6$ breakdown. However, documentation of the effects of free or released MI in poultry is scarce. Because plant seeds and by-products are major components of poultry diets and P is of specific economic and environmental relevance, the improvement of the digestibility of plant P in poultry is of great interest.

The overarching aim of this thesis was therefore to gain a deeper insight into the degradation of inositol phosphates (InsPs) in broiler chickens, with a focus on the intermediate and end-products as influenced by the diet composition. This was investigated with *in vitro* and *in vivo* experiments.

Aiming to reduce the number of animals used for *in vivo* trials, an *in vitro* assay was established to study the disappearance of $InsP_6$ and the formation of lower InsP isomers in the digestive tract of poultry (MANUSCRIPT 1). The assay simulates the conditions (pH, temperature, proteolytic enzymes, water content, and retention time) of the crop, stomach, and small intestine, using a poultry diet as matrix. In this *in vitro* assay, the influence of diets with different ingredients and additives on InsP degradation was investigated. The assay yielded highly reproducible results with coefficients of variation ≤ 10% for an InsP isomer concentration ≥ 0.4 µmol/g DM (n = 3), and was sensitive to the factors that varied in the experiments. A diminishing effect on InsP degradation was found by the supplementation of P and calcium (Ca). The described assay is a suitable tool that can be used to screen feed enzymes and investigate the effects of supplements in the absence of endogenous phytases.

Based on the outcomes of previous experiments conducted in our working group—where supplementation of P and Ca led to reduced $InsP_6$ disappearance and P digestibility—the first *in vivo* experiment aimed to distinguish between single and interactive effects of P, Ca, and phytase (MANUSCRIPT 2). Effects on $InsP_6$, lower InsP esters, their isomers and MI, and on the prececal digestibility of P, Ca, and amino acids (AAs) in different segments of the digestive tract in broiler chickens were studied. Moreover, a complete picture was drawn of all dephosphorylation steps from $InsP_6$ to MI in the digesta of the terminal ileum. The dietary treatments included diets without (P−, 4.1 g/kg DM) or with (P+, 6.9 g/kg DM) monosodium phosphate supplementation, without (Ca−, 6.2 g/kg DM) or with (Ca+, 10.3 g/kg DM) additional fine limestone supplementation, and without or with 1,500 FTU phytase/kg feed in a factorial design. $InsP_6$ disappearance in the crop was not found to be affected by the addition of Ca or P when phytase was added. Up to the terminal ileum, $InsP_6$ disappearance (P−Ca− 56%) decreased in P+Ca− (40%), and even more so in P+Ca+ (21%), when no phytase was added. Adding phytase removed all effects of P and Ca (77–87%). However, P+Ca+ increased the concentrations of lower InsP esters and reduced free MI in the ileum, even in the presence of phytase. These results indicate that mineral supplements, especially P and Ca combined, reduce the efficacy of endogenous microbial or epithelial phosphatases. Supplementation with phytase increased, while supplementation with Ca decreased the concentration of MI in all segments of the digestive tract and in blood plasma, demonstrating the ability of broilers to fully degrade $InsP_6$ and absorb the released MI. While the prececal AA digestibility was not affected by P and Ca or an interaction between P, Ca, and phytase, it increased with the addition of phytase by 2–6 percentage points. This demonstrates the potential of the phytase used to increase AA digestibility, possibly independent of P and Ca supply.

The objective of the second *in vivo* experiment was to investigate the effects of supplementation with free MI or graded levels of phytase on InsP degradation, concentrations of MI in the digestive tract and blood, bone mineralization, and prececal digestibility of AAs (MANUSCRIPT 3). Birds were fed one of five experimental diets. The control diet—based on wheat, soybean meal, and corn—was calculated to contain adequate levels of all nutrients and was without or with MI supplementation. Three further experimental diets differed in terms of phytase level (modified *E. coli*-derived 6-phytase; 500, 1,500, and 3,000 FTU/kg), with P and Ca levels adapted to the recommendations of the phytase supplier. The gain-to-feed

ratio (G:F) was increased by 0.02 g/g by MI or phytase during the whole experimental period. Prececal P and Ca digestibility, P and Ca concentration in blood serum, and tibia ash weight did not differ between treatments. *Myo*-inositol supplementation was found to lead to the highest MI concentration in the crop, ileum, and blood plasma across treatments. Phytase supplementation increased MI concentrations in the crop and ileum digesta in a dose-dependent manner and in plasma without any dose effect. The prececal digestibility of some AAs was increased by phytase. These outcomes indicate that MI might have been a relevant cause for the increase in G:F. Therefore, it is likely that the release of MI after complete dephosphorylation of phytate is one of the beneficial effects of phytase, along with the release of P and improvement in digestibility of other nutrients. Simultaneously, MI seems to have no diminishing effects on InsP degradation.

It can be concluded that the established *in vitro* assay is a suitable tool to investigate the effects on feed enzymes or differences between feed enzymes in a feed matrix under standardized conditions, without the interference of endogenous phytases or animal-specific variations. Due to the complexity of the processes in the living animal, however, it is not possible to replace *in vivo* experiments completely. Based on the outcome of the present *in vitro* and *in vivo* experiments, the combined supplementation of P and Ca—rather than supplementation of P or Ca solely—seems to be crucial for InsP degradation. To maximize the utilization of PP, P and Ca should be supplemented as little as possible, but without risking a deficiency. The prececal AA digestibility was found to be increased by phytase addition, whereas dietary P and Ca had no significant effect. Also, there is now some evidence that MI can affect the growth and feed efficiency of broiler chickens without affecting InsPs or AAs. As the results regarding effects of P and Ca on $InsP_6$ degradation and phytase effects on AA digestibility are not consistent across studies, and studies investigating the effects of MI are scarce and not consistent, further systematic research is needed.

6 ZUSAMMENFASSUNG

Phosphor (P) ist ein essentielles Element in der Geflügelernährung, das in ausreichender Menge zugeführt werden muss. Aus Pflanzensamen ist er für Nicht-Wiederkäuer jedoch nur teilweise verfügbar, da er dort hauptsächlich als Phytinsäure (*Myo*-Inositol 1,2,3,4,5,6-Hexakisdihydrogenphosphat; $InsP_6$) und deren Salz, dem Phytat, gebunden ist. $InsP_6$-P kann nach schrittweiser Spaltung des P vom Phytatmolekül durch Phytasen (*Myo*-Inositol Hexaphosphat-Phosphohydrolasen) oder Phosphatasen vom Tier genutzt werden. Nach der theoretischen vollständigen Dephosphorylierung von $InsP_6$ stehen dem Tier prinzipiell sechs Phosphatgruppen und das *Myo*-Inositol (MI) zur Verfügung. *Myo*-Inositol hat mehrere biologische Funktionen. Es ist z.B. beim Zellwachstum und -stoffwechsel, beim Fettstoffwechsel und der Insulinsensitivität beteiligt. Neuere Studien vermuten einen Effekt von MI auf die Wachstumsleistung von Broilern, entweder durch freies MI nach Supplementierung oder freigesetzt nach vollständigem $InsP_6$-Abbau. Allerdings sind Studien zu Auswirkungen von freiem oder zugesetztem MI noch spärlich. Da Pflanzensamen und –nebenprodukte Hauptbestandteile von Geflügelfutterrationen sind und P von großer ökonomischer und ökologischer Relevanz ist, ist die Verbesserung der Verfügbarkeit des Pflanzen-P für Geflügel von höchstem Interesse.

Das übergeordnete Ziel dieser Arbeit war es deshalb, einen tieferen Einblick in den Abbau von Inositolphosphaten bei Broilern in Abhängigkeit der Futterzusammensetzung zu gewähren, mit Fokus auf die Zwischenabbau- und Endprodukte. Dies wurde mittels *in vitro*- und *in vivo*-Experimenten untersucht.

Im Bestreben die Tierzahlen in Versuchen zu reduzieren, wurde ein *in vitro* System etabliert, um den $InsP_6$-Abbau und die Bildung niederer InsP-Isomere im Verdauungstrakt des Geflügels zu untersuchen (MANUSKRIPT 1). Das *in vitro* System simuliert die Bedingungen (pH-Wert, Temperatur, Verdauungsenzyme, Wassergehalt und Retentionszeit) in Kropf, Magen und Dünndarm basierend auf einer Futtermatrix. Im *in vitro* System wurde der Einfluss verschiedener Rationen mit unterschiedlichen Komponenten und Zusätzen untersucht. Das System lieferte höchst reproduzierbare Ergebnisse mit Variationskoeffizienten ≤ 10% für InsP Isomer-Konzentrationen ≥ 0.4 µmol/g DM (n = 3) und war sensitiv auf die Faktoren, die in den Versuchen variiert wurden. Die Zulage von P und Calcium (Ca) führte zu einem reduzierten

InsP-Abbau. Das *in vitro* System ist leicht zu handhaben und hat eine hohe Reproduzierbarkeit, was es zu einer schnellen und praktikablen Methode zur Untersuchung des Abbauweges von $InsP_6$ unter standardisierten Bedingungen macht.

Basierend auf den Ergebnissen von vorigen Versuchen unserer Arbeitsgruppe, bei denen die Supplementierung von P und Ca zu vermindertem $InsP_6$-Abbau und reduzierter P Verdaulichkeit führte, war das Ziel des ersten *in vivo* Versuchs die Unterscheidung zwischen Einzel- und Interaktionseffekten von P, Ca, und Phytase (MANUSKRIPT 2). Untersucht wurden Effekte auf niedere InsPs und deren Isomere, sowie auf MI und auf die praecaecale Verdaulichkeit von P, Ca und Aminosäuren (AS) in verschiedenen Abschnitten des Verdauungstrakts des Broilers. Zudem konnte ein vollständiges Bild aller Abbaustufen von $InsP_6$ bis MI im terminalen Ileum dargestellt werden. Die Versuchsrationen umfassten Rationen ohne (P−, 4.1 g/kg DM) oder mit (P+, 6.9 g/kg DM) Mononatriumphosphat-Zulage, ohne (Ca−, 6.2 g/kg DM) oder mit (Ca+, 10.3 g/kg DM) zusätzlicher Futterkalk-Zulage und ohne oder mit 1500 FTU Phytase/kg Futter. Das $InsP_6$-Verschwinden im Kropf wurde nicht durch die Ca- oder P-Zulagen beeinflusst, wenn Phytase zugesetzt wurde. Bis zum terminalen Ileum wurde das $InsP_6$-Verschwinden (P−Ca− 56%) reduziert in P+Ca− (40%) und noch weiter in P+Ca+ (21%), wenn keine Phytase zugesetzt war. Durch Zulage der mikrobiellen Phytase verschwanden diese Effekte (77–87%). Jedoch erhöhte P+Ca+ die Konzentration der niederen InsP-Isomere und reduzierte die MI-Konzentration im Ileum, selbst wenn Phytase zugesetzt war. Diese Ergebnisse weisen darauf hin, dass mineralische Supplemente, vor allem P und Ca in Kombination, die Wirksamkeit von endogenen mikrobiellen oder epithelialen Phosphatasen verringern. Die Zugabe von Phytase erhöhte und die Zugabe von Ca verringerte die Konzentration von MI in allen Verdauungstraktabschnitten und im Blutplasma, was auf die Fähigkeit des Broilers hinweist, $InsP_6$ vollständig abzubauen und freigesetztes MI zu absorbieren. Während die praecaecale AS Verdaulichkeit nicht von den P- und Ca-Zulagen oder einer Interaktion zwischen P, Ca und Phytase beeinflusst wurde, wurde sie durch den Zusatz der Phytase um 2–6 Prozentpunkte erhöht. Dies zeigt das Potential der eingesetzten Phytase auf, die praecaecale AS Verdaulichkeit, unabhängig vom P- und Ca-Gehalt in der Ration, zu erhöhen.

Das Ziel des zweiten *in vivo* Versuchs war es, den Effekt einer Zugabe von freiem MI oder steigenden Konzentrationen einer Phytase auf den InsP Abbau, die Konzentration von MI im

Verdauungstrakt und Blut, die Knochenmineralisierung, und die praecaecale AS Verdaulichkeit zu untersuchen. Die Broiler bekamen jeweils eine von fünf Rationen: Die Kontrollration, mit der sie ausreichend mit allen Nährstoffen versorgt wurden, basierte auf Weizen, Sojaextraktionsschrot und Mais, und war ohne oder mit einer Supplementierung von MI. Drei weitere Versuchsrationen unterschieden sich in ihrem Phytasegehalt (eine modifizierte aus *E. coli* stammende 6-Phytase; 500, 1500 und 3000 FTU/kg), wobei ihr P- und Ca-Gehalt an die Empfehlungen des Phytaseproduzenten angepasst wurden. Die Futtereffizienz (Zunahme:Futteraufnahme) während der gesamten Aufzucht wurde durch die Zugabe von MI oder Phytase um 0,02 g/g gesteigert. Die praecaecale P- und Ca-Verdaulichkeit, die P- und Ca-Konzentration im Blutserum und das Tibiaaschegewicht unterschieden sich nicht zwischen den Behandlungen. Die Zulage von MI hatte verglichen mit den anderen Behandlungen die höchsten MI-Konzentrationen in Kropf, Ileum und Blutplasma zur Folge. Die Phytasezulage erhöhte die MI-Konzentration im Kropf und im Ileum dosisabhängig, und im Plasma ohne dosisabhängige Wirkung. Die praecaecale Verdaulichkeit einiger AS wurde durch die Phytasezulage erhöht. Diese Ergebnisse sind ein Hinweis darauf, dass MI einen relevanten Beitrag zur erhöhten Futtereffizienz geleistet haben könnte. Demnach ist es möglich, dass die Freisetzung von MI nach vollständiger Dephosphorylierung von $InsP_6$ eine der vorteilhaften Wirkungen von Phytasen, neben der Freisetzung von P und der verbesserten Verdaulichkeit anderer Nährstoffe, ist. Gleichzeitig scheint MI keinen Effekt auf den InsP-Abbau zu haben.

Zusammenfassend lässt sich sagen, dass das *in vitro* System ein geeigneter Ansatz ist, um Effekte auf Futterenzyme oder Unterschiede zwischen verschiedenen Futterenzymen in einer Futtermatrix unter standardisierten Bedingungen ohne den Einfluss endogener Phytasen oder tierindividueller Variationen zu untersuchen. Durch die Komplexität der Prozesse im lebenden Tier ist es jedoch nicht möglich, *in vivo* Versuche komplett zu ersetzen. Basierend auf den Ergebnissen aus dem *in vitro* und dem ersten *in vivo* Versuch scheint sich im Wesentlichen die gemeinsame statt einer alleinigen Zulage von P und Ca reduzierend auf den InsP-Abbau auszuwirken. Um die InsP-P-Nutzung zu erhöhen, ist eine reduzierte Supplementierung mit P und Ca anzuraten, ohne dabei aber einen Mangel zu provozieren. Die AS-Verdaulichkeit wurde durch die Phytasezulagen erhöht, während P und Ca keinen Einfluss hatten. Es gibt nun einige Hinweise darauf, dass MI das Wachstum und die Futtereffizienz von Broilern positiv beeinflussen kann. Da sich die Ergebnisse bezüglich der Effekte von P und Ca auf den $InsP_6$-

Abbau und bezüglich Phytaseeffekten auf die AS-Verdaulichkeit in der Literatur sehr unterscheiden und Studien zur Wirkung von MI noch sehr spärlich sind, sind nun weitere systematische Studien nötig.

7 ANNEX

ANNEX 1. Results of experiments investigating the effect of a phytase supplementation on prececal amino acid digestibility in broiler chickens. All studies used corn, soybean, or wheat (or a combination) as main ingredient. Birds were either male or unsexed. Measurement of the prececal amino acid digestibility was done with the marker method. Results were limited to the use of a phytase supplementation between 350 and 3500 FTU/kg.

			Composition of diets					Results		
Reference	**Experimental period**	**Replicates**	**Main components**	**P[1]**	**Ca[1]**	**CP[1]**	**Phytase activity[1]**	**ΔFI[2]**	**pcD AA[3]**	**Phytase effect[4]**
				%			FTU/kg	g	%	Percentage points min-max (mean)
Amerah *et al.* (2014)	d5-21	6	C/SBM	0.5 (0.3 PP)	0.5	22.0[5]	1,000[5] α	+43[6]	72.6 - 93.6	4.1 - 15.1 (9.0)
			C/SBM	0.5 (0.3 PP)	0.7	22.0[5]	1,000[5] α	+86[6]	69.3 - 93.7	6.3 - 17.5 (13.0)
			C/SBM	0.5 (0.3 PP)	0.9	22.0[5]	1,000[5] α	+167[6]	65.1 - 92.5	2.1 - 11.0 (5.6)
			C/SBM	0.5 (0.3 PP)	1.3	22.0[5]	1,000[5] α	+149[6]	67.1 - 91.5	6.7 - 13.4 (11.0)
Camden *et al.* (2001)	d1-21	5	C/SBM	0.6 (0.3 PP)	0.8[5]	-	500[5] β	+88[6]	82.3 - 94.1	-1.0 - 2.9 (1.5)
			C/SBM	0.6 (0.3 PP)	0.8[5]	-	1,000[5] β	+97[6]	83.3 - 94.5	0.0 - 3.5 (2.3)
			C/SBM	0.6 (0.3 PP)	0.8[5]	-	500[5] χ	+62[6]	82.0 - 94.2	-1.3 - 3.1 (1.7)
Centeno *et al.* (2007)	d21-42	6	C/SBM	0.6 (0.3[5] avP)	1.0	18.9	500[5] χ	+73[6]	71.4 - 86.4	-3.1 - 1.8 (-1.7)
and Viveros *et al.* (2002)			C/SBM	0.5 (0.1[5] avP)	1.1	18.7	500[5] χ	+227[6]	75.5 - 90.2	2.9 - 13.2 (9.0)
Chung *et al.* (2013)	d1-22	10	C/SBM	0.6 (0.4 PP)	0.9	20.2[5]	1,944 δ	-7.2[6]	69.6 - 89.9	-3.6 - 2.4 (-0.1)
			C/SBM	0.6 (0.4 PP)	0.9	20.2[5]	492 ε	+40.6[6]	70.5 - 91.1	-2.6 - 5.1 (0.9)
			C/SBM	0.6 (0.4 PP)	0.9	20.2[5]	984 ε	+23.8[6]	70.4 - 90.3	-4.8 - 3.8 (0.9)
Dilger *et al.* (2004)	d8-22	8	C/SBM	0.4 (0.1[5] NPP)	0.6	23.2	662 ε	+37[6]	71.0 - 90.1	-0.2 - 2.7 (1.5)
			C/SBM	0.4 (0.1[5] NPP)	0.6	23.2	1,158 ε	+73[6]	72.7 - 90.6	1.0 - 3.5 (2.1)
Gehring *et al.* (2013)	d27-32	6	C/SBM	0.3 NPP	0.7	17.6	947	-	80.9 - 94.7	0.7 - 2.6 (1.5)
			C/SBM	0.3 NPP	0.7	17.6	2,000	-	81.3 - 94.6	0.6 - 3.4 (2.0)
Li *et al.* (2015)	d7-21	8	C/SBM	0.4 (0.2 PP)	0.7	-	568 α	+83.4[6]	76.8 - 93.2	0.1 - 2.9 (1.0)
			C/SBM	0.4 (0.2 PP)	0.7	-	1,004 α	+82.4[6]	79.5 - 94.2	1.1 - 5.6 (2.6)
			C/SBM	0.4 (0.2 PP)	0.8	-	546 α	+122.3[6]	78.5 - 93.5	1.8 - 6.2 (3.2)
			C/SBM	0.4 (0.2 PP)	0.8	-	1,047 α	+123.6[6]	81.3 - 94.6	2.9 - 9.0 (4.8)
			C/SBM	0.4 (0.2 PP)	1.0	-	450 α	+158.4[6]	79.0 - 93.5	2.3 - 8.2 (4.3)
			C/SBM	0.4 (0.2 PP)	1.0	-	1,108 α	+178.8[6]	81.1 - 94.4	3.4 - 10.3 (5.7)

continues

ANNEX 1. continued

Reference	Experimental period	Replicates	Composition of diets					Results		
			Main components	P[1]	Ca[1]	CP[1]	Phytase activity[1]	ΔFI[2]	pcD AA[3]	Phytase effect[4]
				%			FTU/kg	g	%	Percentage points min-max (mean)
Liu *et al*. (2016)	d15-36	6	W/SBM	0.4[5] (0.2[5] PP)	0.5[5]	18.5	1,000[5] δ	+10[6]	78.5 - 94.4	-0.4 - 0.6 (0.0)
			W/SBM/MBM	0.4[5] (0.2[5] PP)	0.5[5]	18.4	1,000[5] δ	+50[6]	75.1 - 94.9	0.4 - 7.5 (2.1)
			W/SBM/MBM	0.4[5] (0.2[5] PP)	0.5[5]	19.5	1,000[5] δ	-154[6]	63.2 - 92.1	-4.5 - 9.1 (-1.4)
Manangi *et al*. (2009a) and Manangi *et al*. (2009b)	d1-21	4	C/SBM	0.5 (0.3 PP)	0.8	21.7	422 ε	+73[6]	68.3 - 90.2	-3.7 - 1.6 (-1.1)
			C/SBM	0.5 (0.3 PP)	0.8	21.7	976 ε	+97[6]	70.7 - 91.1	-4.6 - 4.8 (-0.1)
			C/SBM	0.5 (0.3 PP)	0.8	21.7	1,226 ε	+68[6]	73.8 - 93.1	-0.3 - 7.1 (1.3)
			C/SBM	0.5 (0.3 PP)	1.0	21.1	649 ε	+102[6]	70.2 - 91.4	-2.8 - 1.1 (-1.1)
			C/SBM	0.5 (0.3 PP)	1.0	21.1	934 ε	+190[6]	67.6 - 90.2	-2.7 - 1.3 (-0.2)
			C/SBM	0.5 (0.3 PP)	1.0	21.1	1,075 ε	+138[6]	64.5 - 91.5	-4.6 - 1.6 (-0.1)
	d22-42	4	C/SBM	0.4 (0.3 PP)	0.7	19.3	360 ε	+465[6]	67.9 - 86.6	-1.9 - 1.6 (0.6)
			C/SBM	0.4 (0.3 PP)	0.7	19.3	551 ε	+440[6]	72.4 - 87.5	0.5 - 6.0 (1.9)
			C/SBM	0.4 (0.3 PP)	0.7	19.3	918 ε	+467[6]	71.9 - 87.2	-0.1 - 7.4 (1.7)
			C/SBM	0.4 (0.3 PP)	0.7	19.3	1,246 ε	+200[6]	72.2 - 90.8	3.8 - 9.2 (6.1)
			C/SBM	0.4 (0.3 PP)	0.8	19.0	730 ε	+773[6]	65.9 - 85.8	-5.7 - 4.5 (-0.8)
			C/SBM	0.4 (0.3 PP)	0.8	19.0	900 ε	+770[6]	69.2 - 88.0	-2.7 - 7.7 (1.7)
			C/SBM	0.4 (0.3 PP)	0.8	19.0	1,073 ε	+530[6]	77.2 - 86.9	-4.1 - 16.1 (3.3)
Namkung and Leeson (1999)	d1-15	12	C/SBM	0.6 (0.4[5] avP)	0.8	-	1,149 χ	-10.2[6]	67.0 - 91.3	-1.0 - 4.1 (1.6)
Olukosi *et al*. (2012)*	d42-48	8	C/SBM	0.4	0.8	19.9	489		81.7 - 92.8[8]	-0.4 - 2.1 (1.3)
			C/SBM	0.4	0.8	19.9	489		77.4 - 91.7[9]	-1.2 - 3.5 (1.7)
			C/SBM	0.4	0.8	19.9	899		78.4 - 92.1[8]	-3.0 - 0.8 (-1.1)
			C/SBM	0.4	0.8	19.9	899		78.5 - 92.2[9]	-0.7 - 3.6 (1.9)
Onyango *et al*. (2005)*	d8-22	6	C/SBM	0.4 (0.2 PP)	0.7	26.6	439 ϕ	+62[6]	67.6 - 90.7	-2.5 - 1.2 (0.1)
			C/SBM	0.4 (0.2 PP)	0.9	26.3	825 ϕ	+85[6]	69.0 - 90.7	-1.2 - 2.1 (0.8)
Pieniazek *et al*. (2017)	d1-21	12	C/SBM	0.9 (0.2[5] avP)	1.0	20.0[5]	412 γ	+366[6]	63.0 - 96.8	1.6 - 10.5 (6.2)
			C/SBM	0.9 (0.2[5] avP)	1.0	20.0[5]	1,738 γ	+488[6]	63.7 - 96.9	1.5 - 11.2 (5.8)
	d29-42	11	C/SBM	0.4 (0.2[5] avP)	0.9	18.5[5]	430 γ	+249[6]	59.6 - 88.9	-5.2 - 0.5 (-2.5)
			C/SBM	0.4 (0.2[5] avP)	0.9	18.5[5]	2,100 γ	+402[6]	73.1 - 91.9	2.3 - 8.3 (4.5)

continues

ANNEX 1. continued

Reference	Experimental period	Replicates	Composition of diets					Results		
			Main components	P[1]	Ca[1]	CP[1]	Phytase activity[1]	ΔFI[2]	pcD AA[3]	Phytase effect[4]
				%			FTU/kg	g	%	Percentage points min-max (mean)
Ravindran *et al.* (1999)	d35-42	3	C	0.3[10] (0.2[10] PP)	-	8.4[10]	1,200[5] χ	-	70.4 - 88.8	0.6 - 4.4 (2.5)
			W	0.2[10] (0.2[10] PP)	-	10.6[10]	1,200[5] χ	-	76.8 - 94.4	3.1 – 10.4 (7.0)
			SBM/glucose	0.7[10] (0.5[10] PP)	-	48.3[10]	1,200[5] χ	-	79.5 - 89.9	2.0 - 5.9 (3.4)
Ravindran *et al.* (2000)	d7-25	5	W/SBM	0.5 (0.3 PP)	0.9	21.5	400[5] χ	-	76.9 - 88.2	2.4 - 6.5 (4.8)
			W/SBM	0.5 (0.3 PP)	0.9	21.5	800[5] χ	-	75.4 - 87.0	1.7 - 4.8 (3.5)
			W/SBM	0.7 (0.4 PP)	1.2	21.4	400[5] χ	-	75.2 - 86.5	0.9 - 1.8 (1.4)
			W/SBM	0.7 (0.4 PP)	1.2	21.4	800[5] χ	-	75.8 - 87.2	1.3 - 2.6 (1.9)
			W/SBM	0.6 (0.4 PP)	1.0	22.1	400[5] χ	-	75.7 - 88.0	2.7 - 5.2 (4.0)
			W/SBM	0.6 (0.4 PP)	1.0	22.1	800[5] χ	-	74.3 - 87.6	1.6 - 3.8 (2.8)
			W/SBM	0.8 (0.5 PP)	1.3	21.8	400[5] χ	-	74.8 - 86.3	0.8 - 2.2 (1.7)
			W/SBM	0.8 (0.5 PP)	1.3	21.8	800[5] χ	-	75.5 - 86.9	0.9 - 2.8 (2.0)
			W/SBM	0.7 (0.5 PP)	1.0	21.8	400[5] χ	-	73.8 - 87.1	2.6 - 4.0 (3.3)
			W/SBM	0.7 (0.5 PP)	1.0	21.8	800[5] χ	-	75.0 - 88.6	3.5 - 5.2 (4.5)
			W/SBM	0.9 (0.6 PP)	1.4	21.3	400[5] χ	-	71.4 - 84.9	0.9 - 1.6 (1.3)
			W/SBM	0.9 (0.6 PP)	1.4	21.3	800[5] χ	-	72.8 - 86.2	1.3 - 3.8 (2.8)
Ravindran *et al.* (2001)	d7-28	6	W/SG/SBM	0.8[5] (0.3[5] PP)	1.0[5]	19.6	378 χ	+34[6]	74.3 - 84.9	-2.6 - 3.9 (1.8)
			W/SG/SBM	0.8[5] (0.3[5] PP)	1.0[5]	19.6	688 χ	+22[6]	78.5 - 85.6	2.6 - 4.7 (3.4)
			W/SG/SBM	0.8[5] (0.3[5] PP)	1.0[5]	19.6	1,068 χ	+17[6]	77.9 - 85.4	1.8 - 5.2 (3.5)
			W/SG/SBM	0.8[5] (0.3[5] PP)	1.0[5]	19.6	1,363 χ	+19[6]	79.1 - 86.2	3.0 - 5.4 (4.4)
Ravindran *et al.* (2006)	d14-21	6	C/SBM	0.5[5] (0.3[5] PP)	0.8	22.0	500[5] ε	-	70.7 - 90.8	-0.3 - 4.6 (1.0)
			C/SBM	0.5[5] (0.3[5] PP)	0.8	22.0	750[5] ε	-	73.2 - 91.8	1.3 - 7.1 (2.8)
			C/SBM	0.5[5] (0.3[5] PP)	0.8	22.0	1,000[5] ε	-	74.6 - 92.2	2.5 - 8.5 (3.9)
			C/SBM	0.6[5] (0.3[5] PP)	0.8	22.0	500[5] ε	-	70.6 - 91.4	1.5 - 4.7 (3.5)
			C/SBM	0.6[5] (0.3[5] PP)	0.8	22.0	750[5] ε	-	71.3 - 90.6	1.4 - 4.3 (3.1)
			C/SBM	0.6[5] (0.3[5] PP)	0.8	22.0	1,000[5] ε	-	71.5 - 91.3	1.7 - 4.5 (3.3)
			C/SBM	0.7[5] (0.4[5] PP)	0.9	22.2	500[5] ε	-	73.9 - 91.9	2.5 - 9.8 (5.9)
			C/SBM	0.7[5] (0.4[5] PP)	0.9	22.2	750[5] ε	-	72.9 - 91.8	2.4 - 8.8 (5.6)

continues

ANNEX 1. continued

Reference	Experimental period	Replicates	Composition of diets					Results		
			Main components	P[1]	Ca[1]	CP[1]	Phytase activity[1]	ΔFI[2]	pcD AA[3]	Phytase effect[4]
				%			FTU/kg	g	%	Percentage points min-max (mean)
Ravindran *et al*. (2006)			C/SBM	0.7[5] (0.4[5] PP)	0.9	22.2	1,000[5] ε	-	72.4 - 91.7	2.3 - 8.3 (5.3)
Rodehutscord *et al*. (2004)	d14-21	7	C/Cstarch/RSM	-	-	17	450 ζ	-	69 - 94	0 - 3 (1)
			C/Cstarch/RSM	-	-	19.2	450 ζ	-	73 - 94	1 - 3 (2)
			C/Cstarch/RSM	-	-	21.2	570 ζ	-	71 - 93	1 - 3 (1)
			C/Cstarch/RSM	-	-	23.6	470 ζ	-	73 - 93	0
			C/Cstarch/RSM	-	-	25.6	500 ζ	-	70 - 91	-1 - 1 (0)
Rutherfurd *et al*. (2004)	d21-28	10	C/SBM	0.7 (0.4 avP)	0.8[5]	19.5	500[5] δ	-	78.0 - 91.6	0.3 - 7.4 (3.7)
			C/SBM	0.6 (0.4 avP)	0.8[5]	18.6	750[5] δ	-	75.2 - 90.2	0.5 - 5.6 (3.1)
Rutherfurd *et al*. (2012)	d1-21	10	C/SBM	0.6 (0.4 PP)	0.9	19.8	1,107 δ	+3.2[7]	75.0 - 91.1	-2.8 - 12.4 (2.4)
			C/SBM	0.6 (0.4 PP)	0.9	20.0	2,215 δ	+2.2[7]	72.0 - 91.5	-5.8 - 7.9 (2.6)
Santos *et al*. (2008)	d14-21	6	C/SBM	0.6 (0.3[5] PP)	0.8	21.6	500[5] ε	-	78.1 - 94.6	-0.3 - 10.9 (4.7)
			C/SBM	0.5 (0.3[5] PP)	0.8	21.2	750[5] ε	-	78.0 - 94.1	2.5 - 7.5 (4.4)
			C/SBM	0.4 (0.3[5] PP)	0.7	21.1	1,000[5] ε	-	69.6 - 92.7	-1.8 - 2.6 (0.4)
	d28-35	6	C/SBM	0.5 (0.3[5] PP)	0.7	18.4	500[5] ε	-	82.0 - 94.5	-7.4 - 4.1 (1.4)
			C/SBM	0.5 (0.3[5] PP)	0.7	18.3	750[5] ε	-	83.7 - 95.0	-2.0 - 6.5 (2.7)
			C/SBM	0.4 (0.3[5] PP)	0.6	18.3	1,000[5] ε	-	80.2 - 93.6	-0.1 - 6.8 (2.6)
Sebastian *et al*. (1997)	d1-28	3	C/SBM	0.6 (0.5[5] avP)	1.0	22.8	600[5] χ	+40[6]	81.1 - 92.4	0.8 - 6.9 (3.1)
			C/SBM	0.5 (0.3[5] avP)	1.1	22.7	600[5] χ	+87[6]	73.0 - 87.9	-6.9 - -0.7 (-4.2)
			C/SBM	0.4 (0.3[5] avP)	0.6	22.4	600[5] χ	+87[6]	75.2 - 91.3	-6.1 - -0.9 (-3.0)
Selle *et al*. (2003)	d7-28	6	W/SG/SBM	0.8 (0.3 PP)	1.0	19.8	500[5] χ	-7[6]	78.1 - 91.2	0.2 - 4.6 (2.8)
Selle *et al*. (2009b)	d1-21	6	W/SBM	0.6[5] (0.3[5] PP)	0.7[5]	22.0[5]	500[5] ε	+143[6]	69 - 90	-2 - 9 (4)
Truong *et al*. (2015)	d1-40	8	C/SBM	0.5 (0.2 PP[a])	0.6	17.8[a]	769	-	86.1 - 97.0	2.2 - 9.7 (6.2)
Walk *et al*. (2012c)	d0-16	16	C/SBM	0.6 (0.3[5] avP)	1.0	21.8	500[5] ϕ	-1[6]	76 - 89	1 - 2 (2)
			C/SBM	0.6 (0.3[5] avP)	0.6	22.4	500[5] ϕ	-11[6]	75 - 88	-3 - 0 (-1)
Wu *et al*. (2017)	d1-19	6	W/SBM	0.5 (0.2[5] avP)	0.6[5]	20.3	1,114 ϕ	+166[6]	69.3 - 89.2	-2.3 - 5.8 (1.9)
			SG/SBM	0.4 (0.2[5] avP)	0.7[5]	21.5	693 ϕ	+280[6]	69.8 - 90.2	3.2 - 14.8 (7.3)

continues

ANNEX 1. continued

Reference	Period	Replicates	Composition of diets					Results		
			Main components	P[1]	Ca[1]	CP[1]	Phytase activity[1]	ΔFI[2]	pcD AA[3]	Phytase effect[4]
				%			FTU/kg	g	%	Percentage points min-max (mean)
Zhang *et al.* (1999)	d1-49	7	C/SBM	0.4[5] avP	0.7[5]	-	600 χ	+246[6]	82.2 - 92.6	0 - 1.2 (0.4)
MANUSCRIPT 2*	d15-27	7	C/SBM	0.5 (0.3 PP)	0.6	25.0[5]	1,150 φ	+5[7]	58 - 86	2 - 7 (4.6)
			C/SBM	0.4 (0.3 PP)	1.0	25.0[5]	1,150 φ	+17[7]	59 - 85	3 - 7 (4.9)
			C/SBM	0.7 (0.3 PP)	0.6	25.0[5]	1,160 φ	0[7]	59 - 85	1 - 6 (2.1)
			C/SBM	0.7 (0.3 PP)	1.0	25.0[5]	1,230 φ	+3[7]	60 - 85	1 - 4 (2.7)
MANUSCRIPT 3*	d1-22	8	W/SBM/C	0.6 (0.2 PP)	0.8	24.3[5]	361 φ	-1[7,11]	78 - 93	0 - 2 (1)[11]
			W/SBM/C	0.6 (0.2 PP)	0.8	24.3[5]	1,870 φ	-2[7,11]	79 - 93	1 - 3 (2)[11]
			W/SBM/C	0.6 (0.2 PP)	0.8	24.3[5]	3,110 φ	-2[7,11]	79 - 93	1 - 3 (1)[11]

avP, available P; C, corn; MBM, meat-and-bone meal; NPP, non-phytate P; PP, phytate-P; RSM, rapeseed meal; SBM, soybean meal; SG, sorghum; W, wheat

α, Axtra Phy; β, Finnfeed; χ, Natuphos; δ, Ronozyme; ε, Phyzyme; ϕ, *E. coli* phytase; γ, Cibenza Phytaverse; φ, Quantum Blue; ζ, Consensus phytase

[1]Analyzed, unless indicated otherwise

[2]Difference between feed intake (FI) in the respective phytase supplemented treatment and the treatment without phytase supplementation

[3]Minimum and maximum of prececal digestibility (pcD) of amino acids (AAs) across all analyzed AAs

[4]'Phytase effect' indicates the change in percentage points due to phytase supplementation across all analyzed AAs

[5]Calculated values

[6]Feed intake was calculated for experimental period

[7]Feed intake was calculated as average daily feed intake

[8]Calculated with TiO_2 as indigestible marker

[9]Calculated with Cr_2O_3 as indigestible marker

[10]Concentration measured in the feedstuff, not in complete diet

[11]Compared to a control diet with P and Ca calculated to be sufficient

*Analyzed/calculated values in % in DM

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