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Influence of benzoic acid and phytase in low-phosphorus diets on bone characteristics in growing-finishing pigs[1]

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ABSTRACT: In 2 simultaneous experiments (Exp. 1 and Exp. 2), the effects of benzoic acid (BA) and phytase (Phy) in low-P diets on bone metabolism, bone composition, and bone stability in growing and growing-finishing pigs were examined. Exp. 1 was conducted with 16 crossbred gilts in the weight range of 25 to 66 kg BW, whereas in Exp. 2, 32 crossbred gilts (25 to 108 kg BW) were used. All pigs were individually housed in pens and restrictively fed one of 4 diets throughout the experiment. Total P content of the wheat-soybean diets was 4 g/kg (all values as-fed basis). The experimental diets were: 1) CC, unsupplemented control diet; 2) CBA, control diet with 0.5% BA; 3) PhyC, Phy diet with 750 Phy unit (FTU) Phy/kg and no BA; and 4) PhyBA, control diet with 750 FTU Phy/kg and 0.5% BA . Blood samples were taken at the beginning of the experiment, wk 3 (only for pigs in Exp. 1), wk 6, and before slaughter to determine P and Ca in serum and concentrations of total alkaline phosphatase (AP), serum crosslaps (SCL, marker for bone resorption), and osteocalcin (OC, marker for bone formation). Ash, P, and Ca contents of bones and bone stability were examined using the left metatarsal bones and tibia of the pigs after slaughter. Benzoic acid did not influence any of the blood variables (P > 0.09). The addition of Phy increased ($P \leq 0.03$) P concentration in serum from 2.71 ± 0.08 to 3.03 ± 0.07 mmol/L at wk 3 and SCL content from 0.39 ± 0.02 to 0.45 ± 0.02 ng/mL at wk 6 and decreased ($P < 0.05$) OC at wk 6 by 160 ng/mL. No long-term effect of diets on serum mineral concentrations, AP, and bone markers in serum could be detected. Benzoic acid negatively affected ($P \leq 0.03$) Ca content in bones and distal bone mineral density, especially in the younger pigs. In diets CBA and PhyBA, these variables were reduced by 6 and 11%, respectively. Throughout the whole growing and finishing period, Phy increased ($P \leq 0.02$) ash, P, and Ca contents in bones by 29.4, 4.8, and 11.6-g/kg DM. Bone mineral density and bone mineral content were higher in diets with Phy ($P \leq 0.03$), as well as breaking strength of tibia (+

22%) and metatarsal bones (+ 27%; $P < 0.01$). The results of this study indicate that for a healthy skeleton, BA should not be used in low-P diets without the addition of Phy.

Key words: benzoic acid, bone, low phosphorus diet, phytase, pig

INTRODUCTION

As a consequence of environmental problems with P in manure, regulations on nutrient management in agriculture were adopted to control the use of inorganic P in farm animal diets (Jongbloed et al., 1999; Deunert et al., 2007). However, for nonruminant animals, it is almost impossible to ingest the required amount of available P in diets of plant origin because the majority of the P is bound in phytate (Eeckhout and De Paepe, 1994). As 75% of P in the body is stored in bones (Poulsen, 2000), low P availability can cause skeletal problems (Nicodemo et al., 1998; Gutzwiller et al., 2007b). When microbial phytases (**Phy**) are added to low-P diets, P availability and thus bone stability are increased (Radcliffe et al., 1998; Liesegang et al., 2005; Veum and Ellersieck, 2008).

Also the use of organic acid in pig diets is of increasing importance mainly due to the ban on antimicrobial performance enhancers in the European Union in 2006. When added to diets with low-P content, organic acids are thought to reduce bone stability. So far, this effect has been observed with fumaric acid (Liesegang et al., 2002) and benzoic acid (**BA;** Gutzwiller et al., 2007a) being more pronounced in the latter. Benzoic acid as a feed additive has been authorized in the European Union since May 2003 [Commission Regulation (**EC**) 877/2003; European Union, 2003] and is now listed in the group of zootechnical additives (EC 1138/2007; European Union, 2007). The acidification of diet and gastrointestinal tract can cause an increased renal mineral excretion and, thus, reduce bone mineralization (Kraut et al., 1986). It is unclear whether

these effects can be avoided with the addition of Phy but results in the study by Radcliffe et al. (1998) indicate such a protective effect. However, the mode of action is unknown. For this reason, the effect of BA and Phy supplementation of low-P diets on bone metabolism (i.e., bone formation and bone resorption), bone composition, and bone stability was examined in growing and growing-finishing pigs.

MATERIALS AND METHODS

The experiments were conducted at the Institute of Animal Science, ETH Zurich, and at the research station "Chamau" of ETH Zurich. The experimental procedures were approved by the official veterinary authority of the cantons of Zurich (Switzerland; ZH 181/2007 for Exp. 1) and of Zug (Switzerland; ZG 44/06 for Exp. 2).

Pigs and Housing

The experiments were carried out simultaneously at the 2 experimental sites with a total of 48 crossbred gilts [Large white x (Landrace x Large white)] originating from the experimental station "UFA Bühl" (Hendschikon, Switzerland). The 16 gilts of Exp. 1 were kept at ETH Zurich. Initial and final BW of these pigs was 25.2 ± 0.5 and 66.0 ± 0.7 kg, respectively. Exp. 2 was conducted with 32 gilts at the research station "Chamau." The pigs of this experiment had an initial BW of 26.1 ± 0.2 kg and a final BW of 108.3 ± 0.9 kg.

The pigs in both experiments were housed in individual pens. The pens allowed olfactorial and some snout to snout contact among adjacent pigs. Each pen at both experimental sites was equipped with a feed trough and free access to water. Pens in Exp. 1 (1.5 x 1.0 m) were provided with wood shavings as bedding and a wooden block for exploration. In Exp. 2, the pens (3.0 x

1.25 m; maximal length is 3 m, and length adaptable to the pig's size) were provided with straw for bedding and behavioral enrichment. The pigs consumed some of the bedding but the amounts were negligible. At both experimental sites, the temperature of the stables was 20 to 22°C. The experimental rooms were equipped with windows (daylight and no light regimen) and artificial light was only used for cleaning and feeding.

One gilt of Exp. 2 was killed by emergency slaughter at a BW of 86.50 kg due to severe walking problems (paralysis) of unknown origin. The data of bone and serum of this pig were within the range of the other data (Grubbs-test, $P > 0.05$), therefore, they were included in the statistical analysis.

Diets and Feeding

Diets and feeding were the same in both experiments. The diets for the experiments were prepared in 1 batch and then distributed to the 2 sites. Pigs were fed one of 4 experimental diets in a 2 x 2 factorial arrangement of treatments: control diet with no supplementation (**CC),** control diet with 0.5% BA (**CBA**), Phy diet with 750 Phy units (**FTU**) Phy/kg and no BA (**PhyC**), and Phy diet with 750 FTU Phy/kg and 0.5% BA (**PhyBA**). Benzoic acid and Phy were provided by a commercial company (VevoVitall and Ronozyme P, respectively; DSM Nutritional Products Ltd, Basel, Switzerland). Feed was offered as pellets at a daily amount of 190 g diet·$BW^{0.569}$. Pigs in Exp. 1 were fed every 24 h at 0900 h, whereas pigs in Exp. 2 were fed half of the daily amount at 0700 and 1900 h each. Water was available ad libitum in both experiments. In wk 7 (BW: 57.4 ± 0.5 kg), the diet in Exp. 2 was changed from grower to finisher diet. Pigs in Exp. 1 only received grower diet.

The diets were based on cereals, peas and soybean expellers (Table 1). With exception of P, the dietary composition was calculated to meet the requirements of growing-finishing pigs (Table 1; Agroscope Liebefeld-Posieux, 2004). The dietary P content was below the recommendations (Agroscope Liebefeld-Posieux, 2004), which were 5.3 g total P and 2.67 g digestible P/kg in the grower period and 4.3 g total and 2.16 g digestible P/kg the finisher period (Table 1). Calcium to P ratio was set to be 1.3:1 in grower and 1.5:1 in finisher diets. To inactivate native Phy, diets were expanded before adding the commercial Phy.

Feed samples were taken at both sites once every third week. Samples were ground to 0.75 mm in a centrifugal mill (Retsch ZM 1, Arlesheim, Switzerland) and analyzed for DM, crude ash, crude fat, CP, NDF, and ADF by the standard procedures adopted in our laboratory (Robertson and Van Soest, 1981; Naumann and Bassler, 1997). Gross energy was analyzed using a bomb calorimeter (Calorimeter C7000, IKA-Werke, GmbH & Co., KG, Staufen, Germany). For the analysis of P and Ca, the feed samples were ashed for 13 h at 550°C. The content of both minerals was determined by colorimetry with an autoanalyzer (COBAS MIRA, Roche-Autoanalyzer, Basel, Switzerland) with commercial kits [CALC 20 (Axon Lab AG, Baden, Switzerland) for Ca and ABX Pentra (Horiba ABX, Montpellier, France) for P]. Analysis of BA was done with reversed phase liquid chromatography and UV-detection (DSM-RES 3-E, DSM Nutritional Products Ltd.). Activity of phytase was measured in an external laboratory (Biopract GmbH, Berlin, Germany) with their standard procedure. One FTU of Phy was defined as the activity that releases 1 µmol inorganic phosphate from 5.0 mM phytate/min at pH 5.5 and 37°C.

The analyzed contents of grower and finisher diets, respectively, were 88.5 ± 0.1 and 88.3 ± 0.1 % DM, 212.0 ± 1.6 and 181.1 ± 4.3 g CP/kg DM, 13.6 ± 0.1 and 13.4 ± 0.0 MJ DE/kg as-fed, and 4.5 ± 0.0 and 4.1 ± 0.1 g P/kg DM. These values were close to the calculation (Table 1). The

Ca content was 6.8 ± 0.2 g/kg DM in the diets of the grower period and 7.4 ± 0.2 g/kg DM in the finisher diets. This was slightly higher than intended, increasing Ca:P to 1.51:1 and 1.82:1, respectively. Benzoic acid content was 0 ± 0 g/kg in diets CC and PhyC and 5.2 ± 0.5 g/kg in diets CBA and PhyBA. The diets without Phy also had some Phy activity (174 ± 9 FTU/kg in the grower and 241 ± 86 FTU/kg in the finisher period) but it was markedly lower than in the Phy diets (959 ± 113 and 718 ± 74 FTU/kg, respectively).For unknown reasons, analysis of 1 feed sample of diet PhyBA taken 3 wk after the start of Exp. 2 showed very low levels of BA and Phy. All other feed samples of this experimental diet taken during the experiment were within the expected values. Therefore, no influence on blood profile and bone characteristics was expected and all data of pigs fed diet PhyBA in Exp. 2 were used for statistical analysis.

Collection and Analysis of Samples

Blood samples (5 mL; no anticoagulant) were taken from the jugular vein at arrival of the pigs and before slaughter. Additional sampling times were wk 3 and 6 in Exp. 1 and wk 6 (before start of the finisher period) in Exp. 2. For technical reasons, blood samples of the gilts in Exp. 1 were taken in the morning before feeding and of the gilts in Exp. 2 in the evening 10 h after the last meal. Blood samples were centrifuged (3,000 x g for 10 min at 4°C) 30 min after sampling. Blood serum was analyzed for Ca, P, and total unspecific alkaline phosphatase (**AP;** ALP IFCC, Diatools AG, Villmergen, Switzerland), as well as for the 2 bone markers, serum crosslaps (**SCL**, epitope of the carboxyterminal telopeptide of type I collagen, bone resorption marker) and osteocalcin (**OC**, bone formation marker). The mineral content in the serum was determined with colorimetry with an autoanalyzer as described before. One-step ELISA (Osteometer, Biotech, Copenhagen, Denmark) was used to analyze SCL concentrations. Concentration of OC in serum

was measured with radioimmunoassay using a commercial kit (Nichols Diagnostics, San Juan Capistrano, California).

At a BW of 66.0 ± 0.7 (Exp. 1) and 108.3 ± 0.9 kg (Exp. 2), the gilts were slaughtered in a commercial abattoir by electro-stunning and exsanguinations, and the left tibia and metatarsal (**MT**) bones I to IV were collected. Bones were manually cleaned from attached tissue and frozen until further analysis. For determination of P and Ca, the MT were dried (105°C, 24 h), ashed (550°C, 24 h), and analyzed with the same methods as used for the blood samples.

The tibia were measured at 50% tibia length (midshaft) and at 10% tibia length (distal metaphysis) for bone mineral density (**BMD**) and bone mineral content (**BMC**) with peripheral quantitative computer tomography (pQCT, Stratec XCT 2000 bone scanner, Stratec Medizinaltechnik GmbH, Pforzheim, Germany). Calculation of cortical BMD and BMC was done with automatic computation (cortical mode 2; threshold for cortical bone was > 640 mg/cm^3, and values for trabecular BMD and BMC were peel mode 2 and > 710 mg/cm^3 as threshold for trabecular bone). Breaking strength (the maximal force applied to the bone before its failure) of MT II and III (before ashing) and tibia (after pQCT) was measured with 3 point force application [MT: TA-HD Texture Analyzer and Texture Expert V1.17, maximal force 2.5 kN (Stable Micro Systems Ltd, Surrey, UK); tibia: Zwick Z010 and testXpert V10.11, maximal force 10 kN (Zwick, Ulm, Germany)].

Statistical Methods

Bone and blood data of both experiments were analyzed separately as 2 x 2 factorial with the MIXED procedure of SAS (Version 8.2; SAS Inst. Inc., Cary, NC). The pig was the experimental unit, resulting in n = 4 in Exp. 1 and n = 8 in Exp. 2. Benzoic acid, Phy and their

interaction were the defined effects. The blood data were also evaluated for possible long-term effects of the diet on blood variables. For this analysis, the mixed model procedure was run with the defined effects, diet, sampling time and their interaction. If the main effects resulted in $P < 0.05$, differences were considered to be significant. Data are shown as mean ± SE.

RESULTS

Pig Growth Performance

Neither Phy nor BA had any influence ($P \geq 0.10$) on growth performance of the pigs (data not shown). In Exp. 1, ADG, ADFI, and G:F were 769 ± 14 g/d, 1.59 ± 0.01 kg/d, and 0.48 ± 0.01, respectively. The corresponding values for Exp. 2 were 746 ± 1 g/d, 1.53 ± 0.01 kg/d, and 0.49 ± 0.01 in the grower and 789 ± 11 g/d, 2.50 ± 0.01 kg/d, and 0.32 ± 0.00 in the finisher period.

Blood Variables

Phytase increased P ($P = 0.01$) concentration in serum in wk 3 of Exp. 1 from 2.71 ± 0.08 to 3.03 ± 0.07 mmol/L. However, there was no influence on Ca concentration ($P = 0.052$; Figure 1A, B). Neither in the other sampling times of Exp. 1 nor during the whole Exp. 2 (data not shown), Phy had any effect ($P \geq 0.38$) on mineral concentrations in the blood. In wk 3, AP in pigs of Exp. 1 was reduced from an average of 209 ± 11 to 171 ± 13 FTU/kg in diet PhyBA by the interaction BA x Phy ($P = 0.02$, Figure 2A) but neither BA ($P = 0.60$) nor Phy ($P = 0.87$) themselves had any influence on AP activity. This interaction could not be observed at other sampling times in Exp. 1 and was completely absent in Exp. 2 (data not shown).

The concentration of SCL was increased ($P \leq 0.04$) in the Phy diets. This effect could be observed in wk 6 and at the end of Exp. 1 (Figure 2B) as well as in wk 6 in Exp. 2 (data not shown). On average, SCL in pigs fed diets containing Phy was 16.6% greater than the control groups. Phytase lowered (P = 0.046) the concentration of OC in wk 6 of Exp. 1 from 695.76 ± 59.66 to 535.88 ± 49.79 ng/mL (Figure 2C). There was no such effect in the other sampling times of Exp. 1 or in Exp. 2 (data not shown).

Phytase increased ($P \leq 0.01$) SCL:OC in wk 6 and before slaughter in Exp. 1 (data not shown) but not ($P > 0.05$) in Exp. 2 (data not shown). The SCL:OC for Phy diets in Exp. 1 was elevated by 52% (wk 6) and 61% (before slaughter), respectively, compared to control diets.

The addition of BA did not change any of the blood variables or SCL:OC examined at any time point in Exp. 1 and Exp. 2 ($P \geq 0.09$). There was no diet x sampling time interaction on mineral concentrations, AP, or bone markers in serum in any of the 2 experiments ($P > 0.05$).

Bone Composition

The addition of Phy increased ash and mineral content of MT I to IV ($P \leq 0.02$) in Exp. 1 and 2 (Table 2). Benzoic acid reduced ($P = 0.01$) Ca content in Exp. 1 by 8 g/kg DM but not ash or P, whereas there was no effect BA in Exp. 2 ($P = 0.06$ to 0.65). There were no interactions of BA and Phy ($P = 0.73$).

In Exp. 1, the ash content of dried MT increased from 289 ± 5.2 g/kg DM in the control diets to 329 ± 6.6 g/kg DM in the Phy diets ($P < 0.01$). Similarly, P and Ca concentrations increased from 47.12 ± 0.83 to 53.77 ± 1.90 g/kg DM and from 119.40 ± 2.37 to 135.17 ± 2.52 g/kg DM, respectively ($P < 0.01$) when Phy was included in the diet. In addition, Ca-concentration in Exp. 1 was reduced by 6% due to BA (P = 0.01), but there was no interaction

among the two additives (P = 0.73). In Exp. 2, the increase in ash ($P < 0.01$), P ($P = 0.02$), and Ca ($P < 0.01$) contents in the Phy diets compared to control diets was 19, 3, and 7.5 g/kg DM respectively and no effect of BA was observed ($P = 0.18$). Independent of the age of the pigs, ash, P, and Ca content of the MT increased in the order of CBA < CC < PhyBA < PhyC.

Bone Stability

Total and cortical BMD and BMC of the tibia midshaft were markedly improved with Phy in the diet ($P \leq 0.03$) in both experiments (Table 3). The exception was the cortical BMD in Exp. 2, where no effect ($P = 0.11$) of Phy could be found. Cortical BMD and BMC in pigs fed the Phy supplemented diets in Exp. 1 increased by 4.7 and 21.9%, respectively, in comparison to pigs fed the control diets. The bones of the finishing pigs (Exp. 2) showed a similar improvement between 7% for total BMD and 15% for cortical BMD.

In the medial tibia, BA only affected cortical BMC of growing pigs in Exp. 1 ($P = 0.03$). Compared to pigs fed diets without BA, cortical BMC was reduced from 147 ± 6 to 136 ± 7 mg/cm in pigs fed diets with BA. There was no influence of BA on the stability of the midshaft in Exp. 2.

The total and trabecular BMD and BMC in distal metaphysis of the tibia were affected in Exp. 1 by the addition of Phy to the diet ($P \leq 0.02$; Table 4). In Exp. 2, this effect on the distal bone was only observed for total BMD and BMC ($P \leq 0.01$). Total BMD of growing pigs (Exp 1) fed Phy-diets was about 15% higher than that of the pigs fed the diets without Phy. Regarding BMC, the increase from the control to the Phy diets was 20% for total BMC and 30% for trabecular BMC. In Exp. 2, total BMD and BMC of the distal bone, respectively, was reduced from 408 ± 6 mg/cm^3 and 464 ± 7 mg/cm in Phy diets to 382 ± 8 mg/cm^3 and 410 ± 7 mg/cm in

no phytase diets. In contrast, total BMD and BMC, as well as trabecular BMD, were negatively affected by 0.5% BA ($P \leq 0.03$) but only in Exp. 1. Benzoic acid decreased total BMD and BMC of diets CBA and PhyBA on average by 9% and trabecular BMD by 12% compared to the diets CC and PhyC.

In both experiments Phy improved breaking strength of the tibia and MT II and III ($P < 0.01$; Table 5). In Exp. 1, the tibia of pigs fed diets CC and CBA broke at a force of 1.66 ± 0.05 kN which was less ($P < 0.01$) than the 2.04 ± 0.05 kN measured when Phy was added to the diets. The corresponding values for Exp. 2 were 3.09 ± 0.09 kN and 3.75 ± 0.11 kN, respectively. Benzoic acid reduced breaking strength only in MT of Exp. 2 ($P < 0.05$). MT of pigs fed diets containing BA could support 0.15 kN less than those of pigs fed the other diets. There was no effect of BA on tibia in Exp. 2 or of any bone examined in Exp. 1. No BA x Phy interactions on any of the parameters used to describe bone stability were observed.

DISCUSSION

In this study, almost all response criteria examined in the bones were improved by the addition of Phy, independent of the pig's age. In contrast, BA in low-P diets had an adverse effect on bone composition and bone stability, mainly in younger pigs.

Changes observed in bones being caused by BA were not mirrored by measurements examined in the blood. It is known that serum Ca an P are of limited value to describe bone mineralization (Koch and Mahan, 1986). Activity of AP on the other hand has been reported to correlate with bone stability in growing pigs between 16 and 31 kg BW (Boyd et al., 1983) but not in pigs of the weight range 65 to 95 kg (Koch and Mahan, 1986). It is thus possible that the negative interaction of BA and Phy on AP in wk 3 in Exp. 1 reflected a momentary decline in

bone stability. With a BW of 34.33 ± 0.52 kg, the pigs at this time were only slightly above the weight range examined by Boyd et al. (1983). It has to be taken into consideration that total AP originates from several tissues (Christenson, 1997) and only bone specific AP strongly correlates to bone metabolism (Epstein, 1988). The changes in AP concentration found in this study can thus also hint to changes in the metabolism of other tissues. How the interaction of BA and Phy triggered the distinct reduction of AP is unclear. A possibility that the additives increased P availability and thus decreased AP activity has been described by Boyd et al. (1983). However, in a digestibility study conducted with the same gilts and diets as this study, we could not find any interaction in apparent P digestibility; in fact, it was even decreased by the addition of BA (K. Bühler, unpublished data).

In contrast to what could have been expected, SCL concentration was increased in pigs fed the Phy diets. Generally, this bone marker is reduced when bone stability is increased (Briot and Roux, 2005; Vasikaran, 2008) and in this study, Phy clearly increased BMD, BMC, and breaking strength. Probably, the elevated concentration of SCL was not a sign of decreased bone stability but of accelerated bone turnover. The increased SCL:OC in wk 6 and before slaughter in Exp. 1 is a sign for such an intensified turnover.

It has been claimed that OC is a better predictor of bone stability than AP (Carter et al., 1996) as no bone specific AP was examined. However, Nicodemo et al. (1998) could not find a correlation between OC and bone mineralization, which supports the results in the present study. With exception of wk 6, no effects of any of the additives on OC were found in our study. This is in accordance to the study of Liesegang et al. (2002). Their explanation was that low dietary P content and fumaric acid influenced osteoblasts producing bone specific AP more than those

producing OC. Whether this is the case and whether this is a specific reaction to organic acids in general needs further investigation.

When comparing concentrations of the bone markers, SCL and OC, with the actual bone structure, it has to be taken into consideration that these measurements show a static short-term picture of a highly dynamic system. For example, the concentrations of SCL and OC in several species, including humans, follow a circadian rhythm (Hansson et al., 1974; Lepage et al., 1991; Hassager et al., 1992; Muhlbauer and Fleisch, 1995; Liesegang et al., 1999; Liesegang et al., 2003). It is possible that changes in bone turnover were masked by this rhythm. It is unclear whether such a time dependency can also be found in growing-finishing pigs but it is known from piglets that their OC concentration is the highest after midnight and the lowest around noon (Guo et al., 2000). It is therefore possible that the time intervals for blood samples were too long and thus changes in bone turnover were missed. This would explain why no effect of BA on bone markers in serum was observed, although bone structure was affected in Exp. 1. In addition, the results of this study show that the blood variables were not persistently affected by the diet.

As it has been described in several studies for pigs and poultry (Harper et al., 1997; Liesegang et al., 2005; Payne et al., 2005; Sacakli et al., 2006; Veum and Ellersieck, 2008), this experiment demonstrated that bone composition and bone stability was positively influenced when Phy was added to low-P diets. These effects could be observed in gilts with 66 kg BW as well as in those with 108 kg BW.

Concerning the type of organic acid, it has been described for citric acid and fumaric acid that they can increase the availability of phytate P (Boling et al., 2000; Liem et al., 2008). Citric acid can slightly increase metatarsal ash in young pigs (Boling et al., 2000), but compared to

pigs, the effects observed are stronger in broilers (Boling et al., 2000) and are also described when malic or fumaric acid are used as additives (Liem et al., 2008). In this study, BA decreased Ca, BMD, and BMC, especially in the younger pigs and in the distal bone. Benzoic acid is the only organic acid in pig nutrition which reduces urinary pH through its metabolic end product hippuric acid. This acidification is why more Ca is excreted in urine to re-establish the acid-base balance of the body as it is reported in rats (Kraut et al., 1986). In this study, this effect was observed in the reduced Ca content in MT of the younger pigs. This contradicts the findings of Budde and Crenshaw (2003) who reported that the additional Ca was not of skeletal origin.
So far the negative effects of BA on bone characteristics have only been described by Gutzwiller et al. (2007a) and Hess and Gutzwiller (2008) who fed a N and P reduced (**NPr**)- diet. In the study of Gutzwiller et al. (2007a), the effect of BA on bones in younger pigs was more pronounced than in the study described here. This may be based on the fact that the CP content of about 170 g/kg as-fed in the present study could act as buffer, which was not possible in the NPr-diets with 150 g CP/kg as-fed. Phosphorus and Ca contents of the NPr-diets were similar to those used in this study. Findings that describe strong effects of organic acids on bones in low buffering diets support this theory (Kornegay et al., 1994; Biagi et al., 2003).

It seems that the P content in the diet is the first limiting factor affecting bone stability before the changes induced by BA. In the finisher period of Exp. 2, the pigs consumed on average 0.27 g P/MJ (as-fed), which is close to the recommended P intake (Agroscope Liebefeld-Posieux, 2004). The sufficient P supply in the finisher period could have been the reason that the effects of BA on bone characteristics almost completely disappeared. The limiting effect of P is also supported by the results of a similar study where 1% BA added to normal and high protein diets with sufficient total P had no effect on bone characteristics in finishing pigs (Bühler et al.,

2007). However, it is also possible that the observed differences of BA on bone composition and bone stability between growing and finishing pigs were statistical artifacts caused by the low number of pigs (n = 4 in Exp. 1; n = 8 in Exp. 2).

In the present study, the impact of BA on bone metabolism did not lead to impaired health of the pigs despite being very pronounced in the metaphysis of the bone. However, the pigs used in this study were fed a normal diet until the start of the experiment. Further research is needed with pigs receiving the experimental diets described here from weaning to slaughter as well as with mature sows and boars. With this, long-term effects of such diets on skeletal health can be determined.

In conclusion, only short-term effects of BA or Phy on serum mineral, AP, and bone marker concentrations could be detected. On the other hand, the bones themselves showed signs of reduced stability when BA was added to low-P diets compared with the control groups. Nevertheless, these adverse effects could be almost completely compensated for with the addition of Phy. It was also observed that the negative effect of BA on bone stability disappears with increasing age, whereas the positive effect of Phy on bone stability could also be measured in the finisher pigs. Results of this study indicate the necessity of using Phy in low-P diets supplemented with BA to prevent bone problems.

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Table 1. Ingredients and calculated composition of the experimental diets, as-fed basis[1]

	Grower period				Finisher period			
Item	CC	CBA	PhyC	PhyBA	CC	CBA	PhyC	PhyBA
Supplementation, %								
Benzoic acid	−	0.50	−	0.50	−	0.50	−	0.50
Phytase[2]	−	−	0.12	0.12	−	−	0.12	0.12
Ingredient, %								
Barley	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00
Wheat	30.00	30.00	30.00	30.00	50.00	50.00	50.00	50.00
Peas	20.00	20.00	20.00	20.00	8.00	8.00	8.00	8.00
Soybean expellers	11.00	11.00	11.00	11.00	1.50	1.50	1.50	1.50
Potato protein	1.25	1.25	1.25	1.25	2.50	2.50	2.50	2.50
Molasses	2.00	2.00	2.00	2.00	2.50	2.50	2.50	2.50
Fat	0.50	0.50	0.50	0.50	0.40	0.40	0.40	0.40
Limestone	1.06	1.06	1.06	1.06	1.20	1.20	1.20	1.20
NaCl	0.34	0.34	0.34	0.34	0.35	0.35	0.35	0.35
Monocalcium phosphate	0.05	0.05	0.05	0.05	0.07	0.07	0.07	0.07
L-lysine·HCl	0.24	0.24	0.24	0.24	0.26	0.26	0.26	0.26
DL-methionine	0.14	0.14	0.14	0.14	0.01	0.01	0.01	0.01
L-threonine	0.09	0.09	0.09	0.09	0.06	0.06	0.06	0.06
Celite 545[3]	2.83	2.33	2.71	2.21	2.65	2.15	2.53	2.03
Vitamin/mineral premix[4]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Calculated composition								
CP, %	18.10	18.10	18.10	18.10	14.90	14.90	14.90	14.90
DE, MJ/kg	13.30	13.30	13.30	13.30	13.30	13.30	13.30	13.30
Lys/DE, g/MJ	0.84	0.84	0.84	0.84	0.62	0.62	0.62	0.62
Ca, %	0.52	0.52	0.52	0.52	0.54	0.54	0.54	0.54
Total P, %	0.40	0.40	0.40	0.40	0.35	0.35	0.35	0.35
Digestible P, %[5]	0.14	0.14	0.26	0.26	0.12	0.12	0.24	0.24

[1]CC = no additives; CBA = 0.5% benzoic acid and no phytase; PhyC = 750 phytase unit (FTU) phytase/kg diet and no benzoic acid; PhyBA = 750 FTU phytase/kg diet and 0.5% benzoic acid.

[2]The phytase supplementation was equal to 750 FTU/kg diet.

[3] Benzoic acid and phytase were added at the expense of Celite 545 (acid-washed diatomaceous earth). Celite served as digestibility marker as within this study also nutrient digestibility was measured (K. Bühler, unpublished data).

[4]Supplied per kg of diet: 8,000 IU of vitamin A; 1,000 IU of vitamin D_3; 50 mg of vitamin E; 1.5 mg of vitamin B_1; 3.5 mg of vitamin B_2; 2 mg of vitamin B_6; 15 µg of vitamin B_{12}; 430 µg of vitamin K_3; 10 mg of Ca-pantothenate; 20 mg of niacin; 600 µg of folic acid; 239.5 mg of choline; 175 mg of Fe (as $FeSO_4$); 30 mg of Mn (as manganese oxide); 9.8 mg of Cu (as $CuSO_4$); 750 µg of I as ($Ca(IO_3)_2$); 250 µg of Se (as Na_2SeO_3); and 83.5 mg Zn (as ZnO and $ZnSO_4$).

[5]Based on an estimated P release from phytate of 1.2 g/kg for 750 FTU /kg.

Table 2. Ash (g/kg DM) and mineral content (g/kg DM) in metatarsal bones I to IV[1] at 66 (Exp. 1) and 108 kg BW (Exp. 2)

	Diets[2]					*P*-value[3]		
Item	CC	CBA	PhyC	PhyBA	SEM	BA	Phy	BA x Phy
Exp. 1 (n = 4/treatment)								
Ash	298.9	278.7	334.2	322.9	10.9	0.06	< 0.01	0.58
P	48.2	46.1	55.3	52.3	3.2	0.25	< 0.01	0.85
Ca	123.0	115.8	139.8	130.6	4.0	0.01	< 0.01	0.73
Exp. 2 (n = 8/treatment)								
Ash	358.6	343.7	374.7	365.6	6.8	0.06	< 0.01	0.65
P	55.6	54.7	58.2	58.1	1.5	0.65	0.02	0.75
Ca	149.0	143.9	154.9	152.9	3.3	0.18	< 0.01	0.56

[1]bones were not fat extracted prior to analysis

[2]CC = no additives; CBA = 0.5% benzoic acid, no phytase; PhyC = 750 phytase unit (FTU) phytase/kg diet, no benzoic acid; PhyBA = 750 FTU phytase/kg diet and 0.5% benzoic acid.

[3]Effects of benzoic acid (BA), phytase (Phy) and their interaction (BA x Phy).

Table 3. Bone mineral density (BMD, mg/cm^3) and bone mineral content (BMC, mg/cm)[1] in the midshaft of the tibia at 66 (Exp. 1) and at 108 kg BW (Exp. 2)

	Diets[2]					*P*-value[3]		
Item	CC	CBA	PhyC	PhyBA	SEM	BA	Phy	BA x Phy
Exp. 1 (n = 4/treatment)								
BMD_{tot}[4]	659	592	761	716	35	0.08	< 0.01	0.70
BMC_{tot}	164	153	185	181	6	0.08	< 0.01	0.38
BMD_{cor}	1,088	1,061	1,124	1,123	19	0.33	< 0.01	0.38
BMC_{cor}	136	120	159	153	7	0.03	< 0.01	0.32
Exp. 2 (n = 8/treatment)								
BMD_{tot}	670	641	695	705	21	0.60	0.03	0.32
BMC_{tot}	234	253	272	265	11	0.43	< 0.01	0.08
BMD_{cor}	1,093	1,088	1,108	1,105	12	0.66	0.11	0.91
BMC_{cor}	193	210	235	227	9	0.49	< 0.01	0.054

[1]BMD and BMC were determined with pQCT.

[2]CC = no additives; CBA = 0.5% benzoic acid and no phytase; PhyC = 750 phytase unit (FTU) phytase/kg diet and no benzoic acid; PhyBA = 750 FTU phytase/kg diet and 0.5% benzoic acid.

[3]Effects of benzoic acid (BA), phytase (Phy) and their interaction (BA x Phy).

[4]tot: total, cor: cortical.

Table 4. Bone mineral density (BMD, mg/cm^3) and bone mineral content (BMC, mg/cm)[1] in the distal metaphysis of the tibia at 66 (Exp. 1) and at 108 kg BW (Exp. 2)

	Diets[2]					*P*-value[3]		
Item	CC	CBA	PhyC	PhyBA	SEM	BA	Phy	BA x Phy
Exp. 1 (n = 4/treatment)								
BMD_{tot}[4]	301	264	343	318	18	0.03	< 0.01	0.66
BMC_{tot}	281	265	344	312	11	0.03	< 0.01	0.40
BMD_{trab}	317	277	363	320	18	0.01	< 0.01	0.82
BMC_{trab}	133	125	165	171	29	0.95	0.02	0.67
Exp. 2 (n = 8/treatment)								
BMD_{tot}	378	385	413	402	12	0.87	0.01	0.40
BMC_{tot}	404	416	473	454	11	0.75	< 0.01	0.13
BMD_{trab}	354	354	373	363	10	0.56	0.11	0.56
BMC_{trab}	299	295	310	300	14	0.55	0.49	0.78

[1]BMD and BMC were determined with pQCT.

[2] CC = no additives; CBA = 0.5% benzoic acid and no phytase; PhyC = 750 phytase unit (FTU) phytase/kg diet and no benzoic acid; PhyBA = 750 FTU phytase/kg diet and 0.5% benzoic acid.

[3]Effects of benzoic acid (BA), phytase (Phy) and their interaction (BA x Phy).

[4]tot: total, trab: trabecular.

Table 5. Breaking strength (kN) of tibia and metatarsal bones (MT) II + III at 66 (Exp. 1) and 108 kg BW (Exp. 2)

	Diets[1]					*P*-value[2]		
Item	CC	CBA	PhyC	PhyBA	SEM	BA	Phy	BA x Phy
Exp. 1 (n = 4/treatment)								
Tibia	1.68	1.63	2.09	1.99	0.11	0.34	< 0.01	0.72
MT	0.54	0.46	0.69	0.65	0.06	0.18	< 0.01	0.71
Exp. 2 (n = 8/treatment)								
Tibia	3.05	3.13	3.88	3.61	0.16	0.54	< 0.01	0.24
MT	1.13	0.97	1.33	1.19	0.10	0.048	< 0.01	0.90

[1]CC = no additives; CBA = 0.5% benzoic acid and no phytase; PhyC = 750 phytase unit (FTU) phytase/kg diet and no benzoic acid; PhyBA = 750 FTU phytase/kg diet and 0.5% benzoic acid.

[2]Effects of benzoic acid (BA), phytase (Phy) and their interaction (BA x Phy).

Figure 1: Mean (± SE) serum calcium (A) and phosphorus (B) concentrations (mmol/L) of the 4 groups in Exp. 1. CC = no additives; CBA = 0.5% benzoic acid and no phytase; PhyC = 750 phytase unit (FTU) phytase/kg diet and no benzoic acid; PhyBA = 750 FTU phytase/kg diet and 0.5% benzoic acid. n = 4. Serum was collected at arrival, wk 3, wk 6, and before slaughter at a BW of 66 kg.

Figure 2: Mean (± SE) A) serum alkaline phosphatase (AP) activities (IU/L), B) serum crosslaps (SCL) and C) osteocalcin (OC) concentrations (ng/mL) of the 4 groups in Exp. 1. CC = no additives; CBA = 0.5% benzoic acid and no phytase; PhyC = 750 phytase unit (FTU) phytase/kg diet and no benzoic acid; PhyBA = 750 FTU phytase/kg diet and 0.5% benzoic acid. n = 4. Serum was collected at arrival, wk 3, wk 6, and before slaughter at a BW of 66 kg.

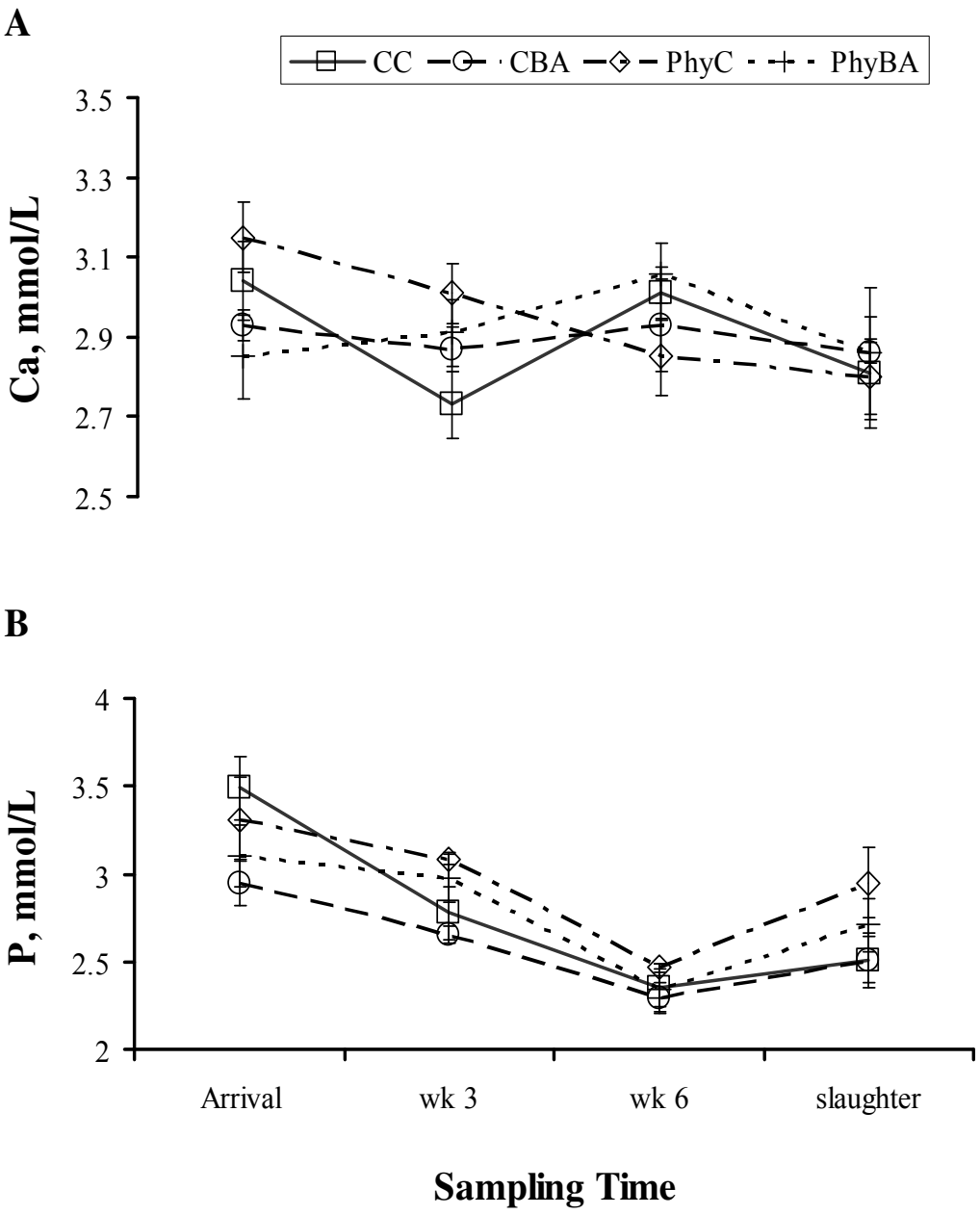
A
CC
CBA
PhyC
PhyBA
Ca, mmol/L
3.5
3.3
3.1
2.9
2.7
2.5
B
P, mmol/L
4
3.5
3
2.5
2
Arrival
wk 3
wk 6
slaughter
Sampling Time

Figure 1

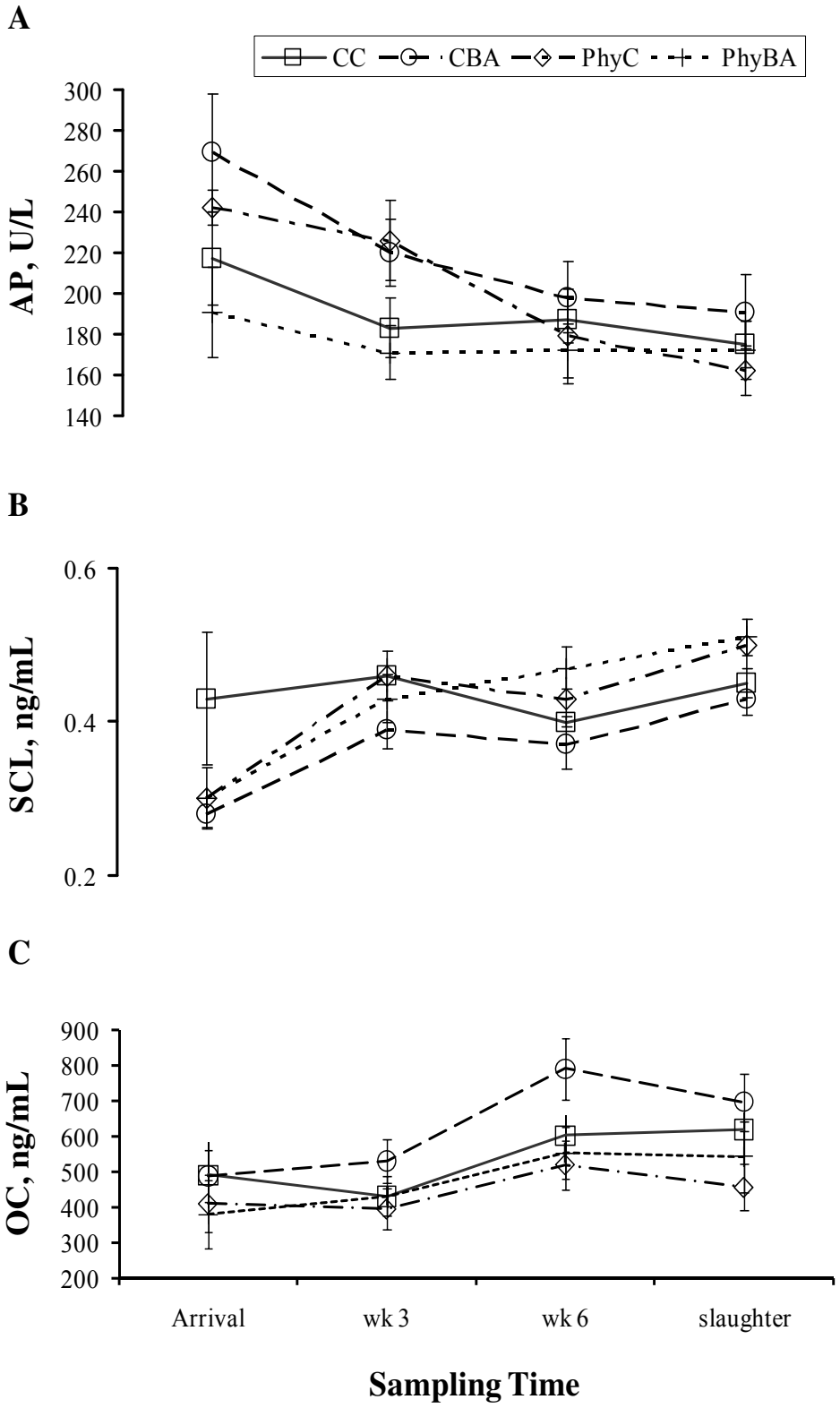
A
CC
CBA
PhyC
PhyBA
AP, U/L
300
280
260
240
220
200
180
160
140
B
SCL, ng/mL
0.6
0.4
0.2
C
OC, ng/mL
900
800
700
600
500
400
300
200
Arrival
wk 3
wk 6
slaughter
Sampling Time

Figure 2