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Effects of a reduced calcium, phosphorus and protein intake and of benzoic acid on calcium and phosphorus metabolism of growing pigs

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ABSTRACT

In order to minimise environmental pollution, many pig feeds contain low phosphorus and protein concentrations as well as benzoic acid (BA), an additive which reduces ammonia formation in the slurry. Since both a low P intake and metabolic acidosis compromise bone mineralisation, the effect of a diet with a low concentration of calcium (Ca), phosphorus (P) and crude protein (CP) and the effect of BA on Ca and P metabolism were examined in a 2 × 2 two-factorial experiment using pigs from 13 to 64 kg body weight (BW). Compared to the control piglet and grower diets (8.7 and 6.9 g Ca; 6.9 and 5.3 g P; 172 and 156 g CP per kg, respectively), the intake of the low nutrient piglet and grower diets (5.3 g Ca; 4.3 and 4.0 g P; 154 and 147 g CP per kg, respectively, both supplemented with 1500 U/kg microbial phytase) reduced ($P<0.01$) Ca and P retention by 27% and 24%, respectively, reduced ($P<0.05$) the growth rate of the piglets by 7%, and decreased ($P<0.05$) the bone breaking strength and bone mineral content ($P<0.01$) by 5% in the animals which were slaughtered at 64 kg BW. Benzoic acid (5 and 10 g per kg piglet and grower diet, respectively) did not influence ($P>0.05$) the apparent digestibility of Ca, increased the apparent digestibility of P ($P<0.05$) by 5% and increased the urinary Ca and P output ($P<0.01$) by 70% and 83%, respectively, but had no effect ($P>0.05$) on the proportion of ingested Ca and P which was retained. Furthermore, BA increased ($P<0.01$) the serum activity of the bone formation marker alkaline phosphatase at 25 and 40 kg BW by 17% and 13%, respectively and decreased ($P<0.01$) the concentration of the bone resorption marker serum crosslaps at 25 kg BW by 12%, implying that BA affected bone metabolism at 25 and 40 kg BW. Since BA neither affected the blood variables at 60 kg nor the bone breaking strength and bone mineral content, any possible negative effect of BA on bone metabolism of the piglets and of the young growing animals thus seems to have disappeared during the last period of the grower period. In conclusion, the slight metabolic acidosis caused by BA had no lasting negative effects on the bones of the growing pigs.

Abbreviations: ADG, average daily weight gain; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; BA, benzoic acid; BMC, bone mineral content; BW, body weight; CP, crude protein; DE, digestible energy; dEB, dietary electrolyte balance; DM, dry matter; GGT, gamma-glutamyltransferase; Mc3, Mc4, 3rd, 4th metacarpal bone; N, Newton; NL, nutrient level; OC, osteocalcin; SCL, serum crosslaps (epitope of the carboxyterminal telopeptide of type I collagen).

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1. Introduction

Pig slurry contributes to the pollution of the environment with phosphorus (P). To minimise P output, the P concentration of feeds for growing pigs is frequently reduced to the lowest levels necessary for maximal growth rate. Since the P requirement for maximal bone mineralisation is higher than the requirement for maximal growth rate (NRC, 1998), such feeds may have an adverse effect on bone mineralisation. Feeds with a reduced P concentration usually contain a low Ca concentration as well, since a wide Ca/P ratio decreases P absorption (NRC, 1998). Ammonia emission from animal manure is an additional environmental problem challenging pig producers. The use of feeds with a low crude protein (CP) concentration and added benzoic acid (BA) reduces ammonia emission. After absorption, benzoic acid is transformed in the liver to hippuric acid which is excreted in the urine and reduces microbial urea decomposition. Benzoic and hippuric acid contribute to the metabolic acid load of the body. Chronic acidosis stimulates bone resorption by osteoclasts and may thus compromise bone mineralisation (Arnett, 2003). It is unclear whether a slight increase in dietary acid load is of practical relevance for bone mineralisation in pigs. Whereas Budde and Crenshaw (2003) failed to detect any negative effect of metabolic acidosis caused by chloride intake on the skeleton of piglets, the intake of BA significantly decreased the bone ash concentration in growing pigs in a balance study (Sauer et al., 2009), and tended to reduce bone ash concentration in growing-fattening pigs (Bühler et al., 2010). One objective of the present experiment was to determine if commercially available Swiss feeds formulated to minimise environmental pollution affect the bone mineralisation and thus compromise the welfare of growing pigs. For this purpose experimental piglet and grower diets with CP, P and phytase concentrations as found in these commercial feeds were formulated, and the effects of these diets were compared to the effects of diets which were formulated to contain the nutrient concentrations recommended in Switzerland by ALP (2004) and which did not contain added phytase. In addition, the hypothesis was tested that the increased dietary acid load provided by BA affects Ca and P metabolism, related blood serum parameters and bone mineralisation of pigs. The effects of dietary nutrient concentration and of BA on Ca and P metabolism, growth and bone traits of pigs were examined in a balance study and a feeding trial. As the focus was set on the period of rapid lean body mass accretion, i.e. the period of high Ca and P requirements and the highest risk of Ca and P deficiency, the animals were slaughtered after attaining 60 kg body weight (BW) in order to study the bone traits at the end of the grower period.

2. Animals, materials and methods

The balance study and the feeding trial were approved by the animal welfare department of the competent government authority (approval number FR 77/06).

2.1. Experimental design and feed formulation

The effects of two factors, dietary nutrient level (Ca, P and CP) and BA supplementation, were examined in a balance study using 16 pigs and in a feeding trial using 64 pigs. Groups of four littermates of the same gender with a similar body weight (BW) were blocked. Each piglet within a block was randomly assigned to one of the four dietary treatments.

The experimental diets contained either a high (H) or a low (L) Ca, P and CP concentration. Benzoic acid was added to the diets H+ and L+, whereas the diets H− and L− contained no BA. The pelleted piglet and grower diets (Table 1) were formulated according to the recommendations for piglets weighing 20 kg and for growing pigs weighing 40 kg (ALP, 2004), except for the Ca, P and CP content of diets L+ and L−, which corresponded to the levels found in commercially available Swiss pig feeds formulated with the aim to minimise P and nitrogen effluent. The Ca/P ratio of all diets was fixed at 1.3/1. The feed formulation programme Allix2 (A-Systems SA, Versailles, France) used to formulate the experimental diets contained the analysed dry matter, crude protein, crude fat, ash and crude fibre data of the ingredients and feed table data of their mineral content and their amino acid composition. Phytase (1500 U/kg feed; Natuphos 5000 G, BASF, Ludwigshafen, Germany) was added to diets L+ and L−. Five and 10 g BA (VevoVitall, DSM Nutritional Products Ltd., Basel, Switzerland) were added per kg of the respective piglet and grower diets (H+ and L+), which corresponds to the dose recommended by the manufacturer.

2.2. Animals and husbandry

Large White piglets, which had been weaned at the age of five weeks and had received a piglet diet containing 180 g CP, 11 g Ca and 7 g P per kg feed during the first two to three weeks after weaning, were selected for the experiments at an average BW of 13 kg. They were housed in a climate controlled building and were fed the experimental piglet diets *ad libitum* until 25 kg BW. Thereafter they received the grower diets in amounts allowing for an average daily gain of 850 g during the growing-finishing period. Drinking water was constantly available.

The sixteen castrated male piglets weighing 13.5 ± 1.8 kg at the start of the balance study were individually housed in pens with a surface of 2.6 m^2.

At a BW of 12.9 ± 2.6 kg the 36 female and 28 castrated male piglets used in the feeding trial were equipped with transponders and were transferred to four pens (one for each treatment) with 7 m^2 of slatted floor and 10 m^2 of concrete floor with straw bedding. Each pen was equipped with a computer controlled feeding station (Schauer, Prambachkirchen, Austria) which registered the amount of the experimental feed consumed by each animal and allowed for individual feed

Table 1
Ingredients and content of the experimental diets (g/kg feed with a dry matter content 0.88 g/g, unless otherwise indicated).

Diets	Piglet diets		Grower diets	
Nutrient level	H	L	H	L
Ingredients				
Maize	200	250	200	250
Barley	137	193	206	226
Wheat	100	50	250	150
Wheat starch	100	125	51	85
Wheat middlings	4	4	4	4
Oats	64			
Fat (tallow and lard)	17	13	3	7
Potato protein	71	98	32	96
Expelled soybean meal	76		12	
Expelled canola meal	30			
Whey powder	50	50		
Casein	10	10		
Dried beet pulp	50	100	50	71
Dried apple pomace	50	74	50	82
L-lysine–HCl	2.8	4.2	3.0	2.9
DL-methionine	1.3	2.1	0.8	0.9
L-threonine	0.9	1.5	0.9	0.6
L-tryptophan	0.4	0.7	0.2	0.4
Dicalcium phosphate	18.4	8.6	11.5	6.1
Calcium carbonate	7.1	4.9	8.1	6.3
Sodium chloride	3.0	4.0	2.7	4.8
Premix[a]	4.0	4.0	4.0	4.0
Phytase, units (U)[b]		1500		1500
Pellet binding aid[c]	3.0	3.0	3.0	3.0
Nutrients				
Crude protein[d]	172	154	156	147
Lysine[e]	12.1	12.1	10.2	10.2
Methionine + Cysteine[e]	7.5	7.5	6.5	6.5
Tryptophan[e]	2.4	2.4	2.0	2.0
Crude fibre[d]	42	42	41	42
Crude fat[d]	36	26	25	25
Ash[d]	52	40	48	42
Ca[d]	8.7	5.3	6.9	5.3
P[d]	6.9	4.3	5.3	4.0
K[e]	5.8	4.3	5.8	3.7
dEB (mEq/kg)[e,f]	121	82	133	82
Phytase activity, U/kg[d]	140	1600	220	1580
Digestible energy (DE)[e], MJ/kg	13.9	13.9	13.6	13.6

Vevovitall (DMS, Geleen, Netherlands) was added to the benzoic acid containing diets (5 g/kg piglet diet and 10 g/kg grower diet) in the feed mill during the feed blending process.

[a] Providing the following amounts of trace elements and vitamins per kg of piglet/grower diet: 40/20 mg Fe; 0.15/0.15 mg I; 6/4 mg Cu; 10/10 mg Mn; 75/55 mg Zn; 0.2/0.15 mg Se; 8000/4000 IU A; 1000/400 IU D3; 25/65 mg E; 3/1 mg K3; 2/2 mg thiamine; 5/3 mg riboflavin; 0.1/0.05 mg biotin; 20/15 mg niacin; 15/15 mg pantothenic acid 300/200 mg choline; 4/3 mg B6; 0.5/0.5 mg folate; 0.02/0.02 mg B12.

[b] Natuphos 5000 G (BASF, Ludwigshafen, Germany).

[c] Pellan (Mikro-Technik, Bürgstadt,Germany).

[d] Analysed.

[e] Calculated using table values for each ingredient.

[f] dEB: dietary electrolyte balance, expressed in milliequivalents (mEq K^+ + mEq Na^+ − mEq Cl^-).

rationing. After attaining 25 kg BW, the pigs used in the feeding trial were transferred to four adjacent identically equipped pens where they were fed the grower diets until they were slaughtered at 64 ± 2.5 kg BW.

2.3. Experimental procedures

2.3.1. Balance study

At the end of each experimental week each pig was weighed and its feed intake during that week was recorded. When the animals were weighing 25, 40 and 55 kg, they were placed into the metabolism crates. From the first collection period onwards, the experimental diets were offered in amounts allowing for an average daily gain of 850 g during the growing-finishing period. During the collection periods lasting five days, faeces, urine and refused feed were collected quantitatively once a day. Faeces and urine samples were stored at −20 °C. At the end of the collection periods, samples of faeces, urine and refused feed were pooled across period for each animal. Faeces were lyophilised for 48 h, and refused feed and faeces were ground in a Brabender laboratory mill using a 1 mm screen.

2.3.2. Feeding trial

The daily feed intake was recorded by the computer controlled feeding stations. The animals were weighed each week, and blood was drawn from the jugular vein of each animal when it had attained 25, 40 and 60 kg BW. The blood samples without added anticoagulant were centrifuged within 1 h after collection, and serum was stored at −20 °C until it was analysed. The 3rd and 4th metacarpal bones and the tibia of the left half of each carcass were collected within half a day after slaughter, and the adjacent tissues were manually removed. The bones were stored in sealed plastic bags at −20 °C until they were analysed.

2.4. Laboratory procedures

Crude protein was analysed using the Dumas method on a Leco FP-2000 analyser (Leco, Mönchengladbach, Germany). Crude fibre, crude fat and ash were analysed according to the VDLUFA methods 6.1.4, 5.1.1 and 8.1. Phytase activity in the feeds was measured using the ISO 30024 method. Briefly, the samples were incubated with sodium phytate, and the reaction was stopped with acid molybdate–vanadate. The complex formed by inorganic phosphate in the presence of molybdate and vanadate was measured photometrically at 415 nm. The 3rd metacarpal bones were crushed, defatted with acetone and dried at 105 °C for the determination of the fat-free dry matter (DM). Samples of defatted bones, feed and faeces were ashed in a muffle furnace at 550 °C until a constant weight was attained. Urine and dry ashed feed, faeces and bones were solubilised in 10 M nitric acid, and their Ca and P concentrations were analysed according to EN 15510:2007 using an inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 2000 DV, Perkin-Elmer, Schwerzenbach, Switzerland).

The serum analytes were assayed using commercially available kits: kit 1489216 for Ca (Roche, Basle, Switzerland; calcium-o-cresolphthalein complexon formation); kit 61571 for P (BioMérieux, Marcy l'étoile, France; absorbance in UV of a phosphomolybdate complex); kit 2172933 for alkaline phosphatase (AP; Roche; cleavage of p-nitrophenyl phosphate); kit 2016788 for gamma-glutamyl transferase (GGT; Roche; formation of 5-amino-2-nitrobenzoate from L-gamma-glutamyl-3-carboxy-4-nitroanilide and glycylglycine); kit 63212 for aspartate aminotransferase (AST; Biomérieux; oxaloacetate formed from aspartate reacting with $NADH_2$); kit 63312 for alanine aminotransferase (ALT; Biomérieux; pyruvate formed from L-alanine reacting with $NADH_2$); Osteometer Biotech (Copenhagen, Denmark; ELISA) for serum crosslaps (SCL, epitope of the carboxyterminal telopeptide of type I collagen); Quidel (San Diego, CA, USA; ELISA) for osteocalcin (OC).

The total bone mineral content (BMC) of the tibia was measured at 50% tibia length (midshaft) and at 10% tibia length (distal metaphysis) using peripheral quantitative computer tomography (pQCT Stratec XCT 960A Bone Scanner; Stratec Medizinaltechnik GmbH, Pforzheim, Germany). The 4th metacarpal bones were transferred from the freezer to a refrigerator 12 h before their breaking strength was determined with a Zwick Roell testing machine (Zwick Roell, Ulm Germany) using the three-point bending test. The bones were held by two supports spaced 35 mm apart and were broken by a wedge lowered on the centre of the bone at a speed of 2 mm/s. The force was measured by a pressure-sensitive cell, and peaks of maximum force (fmax) were recorded.

2.5. Statistical analysis

The data were statistically analysed with the ANOVA procedure of the statistics package NCSS 2007 (Hintze, Kaysville, Utah, USA) using the general linear model. The model included dietary nutrient level (H, L), BA (+, −) and their interaction as fixed factors, and block as random factor. The individual pig was considered the experimental unit. Carcass weight served as a covariate in the ANOVA analysing the results of the bone breaking test. When the interaction between the dietary nutrient level (NL) and BA had a P value <0.05, the means of the four treatments were compared using the Newman–Keuls test. The digestibility and retention data of the balance study as well as the serum Ca, P, AP and SCL data of the feeding trial, which were determined for each animal on three occasions, were further analysed with the repeated-measures ANOVA using the mixed model.

Pearson's linear correlation coefficient *r* was calculated to determine the association among the serum concentration of AP (a bone formation marker) and crosslaps (a bone resorption marker). Differences at $P<0.05$ were considered statistically significant, whereas differences with $0.05 \leq P<0.10$ were considered tendencies.

3. Results

The analysed nutrient content of the experimental diets (Table 1) corresponded to the expected content. The dietary electrolyte balance (dEB) was lower in diets L than in diets H, mainly because diets L contained less potassium (K) and more chloride (Cl) than diets H.

3.1. Balance study

3.1.1. Feed intake

Due to higher feed refusals of the piglets receiving diets L, feed intake during the first balance period at 25 kg BW was 13% lower ($P<0.05$, Table 2) with diets L compared to diets H. At 40 and 55 kg BW, dietary nutrient level had no effect ($P>0.05$)

Table 2
Daily feed intake (kg feed with a dry matter content 0.88 g/g) and urinary pH of pigs at 25, 40 and 55 kg body weight (BW) fed diets with high or low calcium, phosphorus and protein content with or without benzoic acid addition (balance study, $n = 4$)[a].

Nutrient level (NL)	High		Low		S.E.M	P values		
Benzoic acid (BA)	–	+	–	+		NL	BA	NL × BA
Feed intake								
25 kg BW	1.05	1.14	0.88	1.01	0.05	0.01	0.05	0.61
40 kg BW	1.47	1.51	1.24	1.47	0.08	0.14	0.14	0.28
55 kg BW	1.93	1.98	1.80	1.90	0.06	0.09	0.24	0.69
pH of urine								
25 kg BW	5.29[ab]	5.46[ab]	5.72[a]	5.15[b]	0.12	0.57	0.12	0.01
40 kg BW	6.51	5.70	5.85	5.13	0.25	0.03	0.01	0.87
55 kg BW	6.71	5.97	6.21	5.57	0.26	0.11	0.02	0.85

[a] Within row, mean values carrying no common letter differ (P<0.05).

on feed refusals. Benzoic acid tended to reduce (P=0.05) feed refusals at 25 kg BW, but had no effect (P>0.05) at 40 and 55 kg BW.

3.1.2. pH of urine

Addition of BA reduced (P<0.05, Table 2) the urinary pH of the piglets (at 25 kg BW) receiving diet L and of the grower pigs (at 40 and 55 kg BW) receiving diets L and H (P<0.05). The intake of diets L lowered (P<0.05) urinary pH at 40 kg BW.

3.1.3. Mineral balance

Over all three collection periods, the pigs receiving diets L ingested 34% less Ca and 35% less P and had a lower (P<0.01, Tables 3 and 4) faecal Ca and P output than the pigs on diets H, but they retained a higher proportion of the ingested minerals (P<0.01). Despite the higher retention rate (g retained/g ingested), the pigs on diets L retained 27% less Ca and 24% less P (P<0.01) than the pigs on diets H. Over all three collection periods, diets L tended to decrease urinary Ca excretion (P=0.05) and at 25 kg BW they decreased (P<0.05) urinary P excretion. Overall, supplementation with BA increased (P<0.01) urinary Ca excretion but had no effect (P>0.05) on digestibility and retention of Ca. Urinary P excretion was also increased (P<0.01) by BA supplementation. In contrast to Ca, apparent digestibility and retention of P were increased (P<0.05) by BA supplementation,

Table 3
Intake, faecal and urinary excretion, retention (g/d) and apparent total tract digestibility of Ca of pigs at 25, 40 and 55 kg of body weight (balance study, $n = 4$).

Nutrient level (NL)	High		Low		S.E.M	P values		
Benzoic acid (BA)	–	+	–	+		NL	BA	NL × BA
25 kg BW								
Intake	9.47	9.62	4.73	5.37	0.327	<0.01	0.26	0.46
Faecal output	4.61	4.84	1.21	1.87	0.216	<0.01	0.07	0.35
Urinary output	0.08	0.12	0.03	0.06	0.022	0.12	0.36	0.58
Retention	4.79	4.66	3.48	3.44	0.259	<0.01	0.81	0.84
Digestibility	0.51	0.50	0.75	0.66	0.026	<0.01	0.07	0.18
Retention/intake ratio[a]	0.50	0.48	0.74	0.64	0.026	<0.01	0.06	0.18
40 kg BW								
Intake	10.37	10.65	7.13	7.75	0.527	<0.01	0.41	0.76
Faecal output	5.10	4.92	2.26	2.46	0.246	<0.01	0.95	0.46
Urinary output	0.09	0.17	0.05	0.12	0.024	0.10	0.01	0.75
Retention	5.19	5.56	4.82	5.17	0.428	0.40	0.42	0.98
Digestibility	0.51	0.54	0.69	0.68	0.021	<0.01	0.58	0.49
Retention/intake ratio[a]	0.50	0.52	0.68	0.67	0.022	<0.01	0.85	0.51
55 kg BW								
Intake	13.45	13.86	10.15	9.87	0.353	<0.01	0.84	0.36
Faecal output	6.54	6.61	3.27	3.32	0.303	<0.01	0.85	0.98
Urinary output	0.10	0.13	0.08	0.24	0.052	0.39	0.08	0.21
Retention	6.81	7.12	6.80	6.31	0.270	0.16	0.75	0.17
Digestibility	0.51	0.52	0.68	0.66	0.001	<0.01	0.86	0.46
Retention/intake ratio[a]	0.51	0.52	0.67	0.64	0.020	<0.01	0.53	0.31
Overall								
Intake	11.10	11.38	7.21	7.67	0.365	<0.01	0.32	0.81
Faecal output	5.35	5.40	2.21	2.52	0.194	<0.01	0.33	0.47
Urinary output	0.09	0.14	0.06	0.11	0.025	0.05	<0.01	0.91
Retention	5.66	5.84	4.93	5.00	0.233	<0.01	0.59	0.82
Digestibility	0.52	0.52	0.71	0.67	0.014	<0.01	0.28	0.12
Retention/intake ratio[a]	0.51	0.51	0.69	0.65	0.013	<0.01	0.20	0.15

[a] The retention/intake ratio was calculated by dividing the amount of nutrient retained by the amount of nutrient ingested per day.

Table 4
Intake, faecal and urinary excretion, retention (g/d) and apparent total tract digestibility of P in pigs at 25, 40 and 55 kg of body weight (balance study, $n = 4$)[a].

Nutrient level (NL)	High		Low		S.E.M	P values		
Benzoic acid (BA)	–	+	–	+		NL	BA	NL × BA
25 kg BW								
Intake	7.08	7.59	3.71	4.17	0.251	<0.01	0.09	0.98
Faecal output	3.22	3.31	0.85	1.01	0.185	<0.01	0.49	0.81
Urinary output	0.35	0.58	0.17	0.40	0.005	0.01	<0.01	0.81
Retention	3.51	3.69	2.69	2.76	0.206	<0.01	0.54	0.85
Digestibility	0.55	0.56	0.77	0.76	0.028	<0.01	0.98	0.55
Retention/intake ratio[b]	0.50	0.49	0.73	0.66	0.027	<0.01	0.21	0.35
40 kg BW								
Intake	7.65	7.84	4.95	5.79	0.359	<0.01	0.18	0.38
Faecal output	3.80	3.55	1.34	1.38	0.172	<0.01	0.55	0.39
Urinary output	0.18	0.37	0.20	0.41	0.068	0.66	<0.01	0.89
Retention	3.67	3.93	3.41	4.00	0.329	0.79	0.22	0.65
Digestibility	0.50	0.55	0.73	0.76	0.024	<0.01	0.16	0.80
Retention/intake ratio[b]	0.48	0.50	0.69	0.69	0.024	<0.01	0.66	0.72
55 kg BW								
Intake	10.07	10.31	7.16	7.49	0.253	<0.01	0.29	0.87
Faecal output	4.77	4.41	1.93	1.59	0.223	<0.01	0.15	0.98
Urinary output	0.49[b]	0.55[ab]	0.44[b]	1.04[a]	0.125	0.11	0.02	0.05
Retention	4.81	5.34	4.79	4.86	0.218	0.27	0.20	0.30
Digestibility	0.53	0.57	0.74	0.79	0.023	<0.01	0.05	0.87
Retention/intake ratio[b]	0.48	0.52	0.67	0.65	0.019	<0.01	0.66	0.12
Overall								
Intake	8.27	8.58	5.17	5.82	0.261	<0.01	0.08	0.53
Faecal output	3.89	3.72	1.33	1.31	0.144	<0.01	0.40	0.51
Urinary output	0.34	0.50	0.26	0.57	0.054	0.88	<0.01	0.19
Retention	4.13	4.51	3.59	4.00	0.187	<0.01	0.04	0.96
Digestibility	0.53	0.57	0.75	0.77	0.017	<0.01	0.04	0.65
Retention/intake ratio[b]	0.50	0.53	0.70	0.70	0.013	<0.01	0.51	0.26

[a] Different lower case and capital letters as superscripts within a row indicate differences at P<0.05 and <0.01, respectively.
[b] The retention/intake ratio was calculated by dividing the amount of nutrient retained by the amount of nutrient ingested.

but this did not influence (P>0.05) the proportion of ingested P which was retained (retention/intake ratio). An interaction (P=0.05) between nutrient level and BA was observed for urinary P excretion at 55 kg BW, indicating that the effect of BA on urinary P output was more marked at the low dietary nutrient level.

3.2. Feeding trial

3.2.1. Animal performance

The intake of diets L reduced the growth rate (P<0.05) of the piglets by 7%, but neither affected the growth rate during the fattening period (Table 5) nor the daily weight from the beginning of the experiment until slaughter (P>0.05, data not shown). Neither feed intake nor feed conversion was influenced by any treatment.

3.2.2. Serum analytes

The ANOVA for repeated measures shows that the intake of diets L globally increased (P<0.01) the serum Ca concentration and the activity of the bone formation marker AP and tended to decrease (P=0.06) the concentration of the bone resorption

Table 5
Effects of nutrient level and benzoic acid intake on animal performance (feeding trial, $n = 16$)).

Nutrient level (NL)	H		L		S.E.M.	P values		
Benzoic acid (BA)	–	+	–	+		NL	BA	NL × BA
Piglets (13–25 kg BW)								
Initial BW, kg	12.9	12.9	13.0	12.8	0.35	0.96	0.69	0.77
Feed intake, kg/d	0.94	0.94	0.91	0.91	0.026	0.30	0.88	0.88
ADG, g	594	591	539	562	17	0.02	0.57	0.45
Feed conversion, kg/kg	1.68	1.66	1.67	1.73	0.044	0.43	0.55	0.32
Growing pigs (25–60 kg BW)								
Feed intake, kg/d	1.66	1.79	1.68	1.71	0.049	0.54	0.15	0.32
ADG, g	784	818	814	806	13	0.51	0.32	0.12
Feed conversion, kg/kg	2.13	2.18	2.05	2.13	0.046	0.21	0.21	0.84

BW: body weight; ADG: average daily weight gain.

Table 6
Effects of nutrient level and benzoic acid intake on serum analytes and bone traits (feeding trial, $n = 16$)[a].

Nutrient level (NL)	H		L		S.E.M.	P values		
Benzoic acid (BA)	−	+	−	+		NL	BA	NL × BA
Serum analytes								
Overall								
Ca, mmol/l	2.75	2.78	2.85	2.82	0.022	<0.01	0.79	0.07
P, mmol/l	3.15[B]	3.29[A]	3.18[B]	3.20[B]	0.036	0.26	<0.01	0.01
AP, U/l	170	179	177	207	7	0.01	<0.01	0.12
SCL, ng/l	591	569	559	547	19	0.06	0.24	0.71
25 kg BW								
Ca, mmol/l	2.79	2.82	2.91	2.88	0.027	<0.01	0.94	0.26
P, mmol/l	3.24	3.35	3.22	3.26	0.044	0.21	0.09	0.46
AP, U/l	198[B]	211[B]	196[B]	249[A]	10	0.08	<0.01	0.05
SCL, ng/l	545	456	511	476	20	0.76	<0.01	0.23
40 kg BW								
Ca, mmol/l	2.74	2.75	2.83	2.78	0.023	0.01	0.38	0.21
P, mmol/l	3.19[b]	3.33[a]	3.21[b]	3.19[b]	0.035	0.09	0.11	0.02
AP, U/l	167	182	171	201	6	0.07	<0.01	0.23
SCL, ng/l	556	611	544	564	20	0.21	0.11	0.44
60 kg BW								
Ca, mmol/l	2.71	2.77	2.79	2.80	0.02	0.01	0.11	0.17
P, mmol/l	3.02[b]	3.20[a]	3.11[ab]	3.13[ab]	0.03	0.91	<0.01	0.02
AP, U/l	149	150	157	167	7	0.06	0.43	0.48
SCL, µg/l	673	630	620	614	30	0.18	0.33	0.47
Bone traits, tibia								
BMC, midshaft, mg/cm	232	226	212	221	5	0.02	0.79	0.14
Breaking strength, N	770	734	704	717	17	0.04	0.56	0.21

AP: alkaline phosphatase; SCL: serum crosslaps.
[a] Different lower case and capital letters as superscripts within a row indicate differences at P<0.05 and <0.01, respectively.

marker serum crosslaps (SCL) in the serum (Table 6). Diets L+ lowered (P<0.01) the serum P concentration compared to diets H+. Benzoic acid increased serum AP activity (P<0.01) and increased the serum P concentration of the piglets receiving diets H (P<0.01). The effect of BA on the bone turnover markers decreased with advancing body weight: Benzoic acid lowered the SCL concentration at 25 kg BW (P<0.01) only and increased the AP activity at 25 and at 40 kg BW (P<0.01), but not at 60 kg BW. The AP activity and the SCL concentration of the 64 piglets were negatively correlated at 25 kg BW ($r = -0.36$; P<0.01), but not at 40 kg BW ($r = 0.16$; P=0.22) and at 60 kg BW ($r = -0.04$; P=0.77). There was no treatment effect on the concentration of the bone formation marker OC and on the activities of the enzymes AST, ALT and GGT (data not shown).

3.2.3. Bone traits

The intake of diets L reduced the breaking strength (P<0.05) of the Mc4 and the BMC of the midshaft of the tibia by 5% (P<0.05; Table 6), whereas BA intake had no effect on any bone trait. There was no treatment effect on the BMC of the distal tibial epiphysis and on the concentration of ash, Ca and P in the fat free dry matter of the Mc3 (P>0.05, data not shown).

4. Discussion

The pigs receiving the diets L digested Ca and P more efficiently than the pigs on diets H, which can be attributed mainly to the 1500 U phytase added per kg diet L, an amount known to cleave approximately 1 g of P from phytate (Düngelhoef and Rodehutscord, 1995; Kornegay, 2001). The P digestibility coefficient of 0.76 in diets L determined in the balance study is slightly higher than the digestibility coefficient of 0.70 calculated on the basis of tabular values for the ingredients and phytate P hydrolysed by phytase, but is in the order of magnitude observed by Lei et al. (1993a,b), Young et al. (1993), Almeinda and Stein (2010) and Létourneau-Montminy et al. (2010) in pigs fed phytase supplemented diets. Similarly, P digestibility of diets H determined in the balance study was higher than the calculated value (0.55 vs. 0.50 on average). In view of the high variability of P digestibility data of diets containing phytase (Johansen and Poulsen, 2003) and the fact that P digestibility determined by total collection of faeces – the method used in our experiment – often yields higher values than methods using an indigestible marker (Agudelo et al., 2010; Blaabjerg et al., 2010), the experimentally determined P digestibility data correspond quite well to the calculated data. Based on the digestibility values obtained in the balance study, the piglet and the grower diets L contained approximately 0.23 g and 0.22 g digestible P per MJ DE, respectively. The available P requirement for maximum growth of piglets weighing 10–20 kg and of growing pigs weighing 20–50 kg is 3.2 g and 2.3 g per kg diet containing 14.2 MJ/kg DE (NRC, 1998), which corresponds to approximately 0.20 g and 0.15 g digestible P per MJ DE, using the factor 0.9 to convert available P to digestible P (Jongbloed et al., 1991). The P requirement for maximising bone strength and bone ash content is higher than the requirement for growth (NRC, 1998). The digestible P concentration of the experimental piglet diets L thus was not sufficient to maximise bone mineralisation, whereas the grower diets L contained sufficient amounts of digestible P.

This evaluation based on the NRC (1998) data is confirmed by the results of the balance study. The piglets fed diets L retained 27% less Ca and 24% less P than the piglets on diets H, which implies that at 25 kg BW Ca and P requirements for maximum mineral accretion were not met by diets L, whereas retention of Ca and P did not differ between pigs on diets H and pigs on diets L at 40 and 55 kg BW.

Benzoic acid added to the grower diets at 10 g/kg significantly reduced the pH of urine, indicating that the additive markedly increased the dietary acid load. A drop in urinary pH was also observed when 5 g/kg BA were added to the piglet diet L, whereas the buffering capacity of the piglet diet H containing higher concentrations of Ca, P and CP presumably prevented the drop in urine pH.

Benzoic acid increased the apparent digestibility of P but had no effect on the proportion of ingested Ca and P which was retained (retention/intake ratio). By contrast, Mroz et al. (2000), who studied the effects of formic, fumaric and butyric acid and of Ca-benzoate added to feeds with a dEB of 280 meq/kg on growing pigs, observed that 20 g/kg benzoate increased the digestibility and retention/intake ratio of Ca and had no effect on P digestibility, but decreased the retention of P. Sauer et al. (2009) reported that the addition of 10 and 20 g/kg of benzoic acid to the diet of young growing pigs receiving a diets supplemented with sodium bicarbonate (dEB 200 mEq/kg) linearly increased the digestibility and retention/intake ratio of both Ca and P. The reasons for these contradictory observations are unknown but could be related to differences between the experimental diets in their mineral concentration and dEB, the presence of organic acids other than benzoic acid as well as the absence of microbial phytase in the diets used by Mroz et al. (2000) and Sauer et al. (2009).

The increased urinary Ca and P excretion of our pigs receiving diets supplemented with BA is in accordance with the increased urinary P output in pigs ingesting BA reported by Sauer et al. (2009) and the increased urinary output of either Ca alone (Patience and Chaplin, 1997) or of both Ca and P (Budde and Crenshaw, 2003) in pigs fed diets that were acidified with chloride. Pigs thus react similarly as rodents and humans who respond to a metabolic acidosis by increasing urinary Ca and P excretion (Osther, 2006; Novic et al., 2008). Since urinary P output was low compared to P intake and faecal P output, the increased urinary P excretion caused by BA was too low to affect P retention in our pigs. The reduced daily weight gain of the piglets of treatments L was probably caused by the low P intake, since a dietary P concentration slightly below NRC requirement has been shown to reduce the growth rate of piglets without affecting their feed intake (Reinhart and Mahan, 1986). The elevated serum Ca concentration observed in pigs on diets L compared to the pigs on diets H reflect the marginal P intake, a low P status being associated with an increased serum Ca and a decreased serum P concentration in pigs (Reinhart and Mahan, 1986; Scott et al., 1994; Liesegang et al., 2002). It is unclear why in the present trial the serum P concentration decreased in response to a low P intake only in the pigs receiving BA and why BA increased the serum P concentration in piglets receiving diets H exclusively.

The enzyme AP occurs at high concentrations in osteoblasts and liver cells, and an elevated serum activity is a sign of altered osteoblast or liver function. Treatment effects on serum AP in the present experiment can be attributed to altered osteoblast function, since the activities of the enzymes ALT, AST and GGT in the serum, which are used as biomarkers for liver dysfunction, were unaffected by the dietary treatments. The activity of AP is increased in the serum of P deficient growing pigs and is negatively correlated with bone strength (Boyd et al., 1983; Koch and Mahan, 1985; Epke et al., 2002; Liesegang et al., 2002), whereas the concentration of the bone resorption marker SCL is increased in pigs fed a low P diet (Bühler et al., 2010). The increased AP activity and the decreased SCL concentration in the serum of the pigs receiving diets L thus indicates that bone formation and remodelling were affected by the low P intake. The increase in serum AP activity at 25 and 40 kg BW and the decreased SCL concentration at 25 kg BW caused by BA suggests that BA also affected bone metabolism of the young animals, especially of those receiving diets L. No treatment effect on serum OC concentration was observed in our study. OC does not seem to be a sensitive biomarker for altered bone metabolism in P deficient pigs, as in four of six experiments its serum concentration did not change in response to P deficiency (Nicodemo et al., 1998; Liesegang et al., 2002; Bühler et al., 2010; Létourneau-Montminy et al., 2010; vs. Carter et al., 1996; Shaw et al., 2006).

The elevated serum AP activity at 60 kg BW as well as the reduced bone breaking strength and BMC observed in the pigs on diets L imply that these animals were unable to fully compensate the low P intake during the piglet rearing period, although the calculated digestible P content of the grower diets L was above the requirement assessed by NRC (1998). This result supports the finding of Varley et al. (2010a,b) that the bone mineral content of slaughter pigs fed insufficient amounts of P during the weaner period is reduced even if the feed offered later on contains sufficient amounts of P. Maximum incorporation of Ca and P into the bones during the weaner period thus seems to be prerequisite for subsequent maximum bone mineralisation. Although diets L reduced bone breaking strength and BMD, they did not affect the concentration of ash, Ca and P in the bone dry matter of the pigs. The same phenomenon – a significant increase in bone breaking strength, but not in bone P concentration in piglets whose diet was supplemented with phytase – was observed by Columbus et al. (2010). The fact that P deficiency causes osteopenia, that is a reduction in both the ash and the organic matter of the bone (Koch and Mahan, 1985; Hagemoser et al., 2000) decreases the sensitivity of the bone mineral/DM ratio as an indicator of P deficiency. Bone ash/volume or bone P/volume ratio have therefore been recommended to characterise bone mineralisation in P deficient pigs (Hagemoser et al., 2000). Benzoic acid neither affected AP activity at 60 kg BW nor the bone traits. Any possible negative effect of BA on bone metabolism of the piglets and of the young growing animals thus disappeared during the last period of the grower period. The reported effects of BA on bone traits in growing pigs are inconclusive. Increasing amounts of BA added to diets containing the recommended amounts of CP and P had no effect on total bone ash, but decreased the ash concentration in the femoral DM of pigs weighing 40 kg (Sauer et al., 2009), suggesting a possible negative effect of BA on bone mineralisation. Similarly, Bühler et al. (2010) reported that BA tended to reduce ash concentration in the DM of

metatarsal bones of pigs slaughtered at 66 and 108 kg BW. Furthermore, in the same study, BA significantly reduced bone mineral density of pigs slaughtered at 66 kg BW, but had no effect on the bone breaking strength.

In conclusion, the experiments showed that 4.3 g P per kg weaner piglet feed containing 1500 U/kg added phytase is not sufficient for normal Ca and P metabolism and bone mineralisation. Benzoic acid favourably influenced the digestibility of P and had no effect on bone mineralisation at the end of the grower period, but on the other hand affected serum markers of bone turnover at earlier stages of growth. Further studies are necessary to clarify under which conditions BA may affect bone metabolism and if any observed effects are of practical relevance to pig health.

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