Results of an international phosphorus digestibility ring test with broiler chickens

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ABSTRACT The objective of this ring test was to investigate the prececal phosphorus (P) digestibility of soybean meal (SBM) in broiler chickens using the trial protocol proposed by the World's Poultry Science Association. It was hypothesized that prececal P digestibility of SBM determined in the collaborating stations is similar. Three diets with different inclusion levels of SBM were mixed in a feed mill specialized in experimental diets and transported to 17 collaborating stations. Broiler chicks were raised on commercial starter diets according to station-specific management routine. Then they were fed the experimental diets for a minimum of 5 d before content of the posterior half of the ileum was collected. A minimum of 6 experimental replicates per diet was used in each station. All diets and digesta samples were analyzed in the same laboratory. Diet, station, and their interaction significantly affected ($P < 0.05$) the prececal digestibility values of P and calcium of the diets. The prececal P digestibility of SBM was determined by linear regression and varied among stations from 19 to 51%, with significant differences among stations. In a subset of 4 stations, the prececal disappearance of *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate)-P; $InsP_6$-P) also was studied. The prececal $InsP_6$-P disappearance correlated well with the prececal P digestibility. We hypothesized that factors influencing $InsP_6$ hydrolysis were main contributors to the variation in prececal P digestibility among stations. These factors were probably related to the feeding and housing conditions (floor pens or cages) of the birds in the pre-experimental phase. Therefore, we suggest that the World's Poultry Science Association protocol for the determination of digestible P be should extended to the standardization of the pre-experimental period. We also suggest that comparisons of P digestibility measurements among studies are made only with great caution until the protocol is more refined.

Key words: broiler chickens, phosphorus, phytate, prececal digestibility, variability

2017 Poultry Science 96:1679–1687
http://dx.doi.org/10.3382/ps/pew426

INTRODUCTION

Phosphorus (**P**) is an element of high relevance for poultry feeding. Diets are usually supplemented with feed phosphates or phytase or both in order to fulfill the animal's requirement for available P. However, excessive intake can contribute to environmental

Received July 13, 2016.
Accepted November 22, 2016.
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problems in areas with high livestock density. The variation in P availability of different feed raw materials is high (Shastak and Rodehutscord, 2015), and it is generally accepted that the use of P as a globally finite resource can be optimized by considering the differences that exist in P availability of feed raw materials.

Different response criteria and descriptive terms for available P have been used in the literature of the past 7 decades (Shastak and Rodehutscord, 2013). These differences make it difficult to compare results obtained by using different techniques in different laboratories, and to compile comprehensive feedstuff tables needed by the industry. In an attempt to improve this situation the Working Group No 2: Nutrition of the European Federation of Branches of the World's Poultry Science Association proposed a standard protocol for the determination of P availability (WPSA, 2013). This protocol is based on using the digestibility measured at the terminal ileum of broiler chickens (prececal digestibility of P [**pcdP**], otherwise also referred to as ileal digestibility). The protocol defines assay details relevant for the outcome of the measurement, such as age of birds, minimum number of experimental replicates, diet composition, and P and calcium (Ca) levels in the diet.

The expectation from implementing this standard protocol is that results generated in different research stations for the same feed raw material are similar and thus can better be compared and used by the industry. However, application of the standard protocol has not been compared yet among stations. Thus, our objective was to compare the results among our laboratories for one feed raw material when based on the WPSA (2013) standard protocol. We chose soybean meal (**SBM**) for this comparison, and we hypothesized that the pcdP of SBM determined in our laboratories is similar.

MATERIALS AND METHODS

Seventeen research stations from Europe and North America collaborated in this study and determined the pcdP of SBM following the principles of the standard protocol of WPSA (2013), which includes regression analysis. The concept of this ring test required that all stations use the same diets and thus variability in raw material quality and diet preparation was mitigated. Furthermore, chemical analyses of all diets and ileal digesta collected in all stations were conducted in the same laboratory. Customs regulations related to cross-continental shipment of feed samples made it impossible for more laboratories from other regions of the world to participate in the ring test.

Experimental Diets

Three diets that differed in the inclusion level of SBM (diets A, B, C) were formulated based on the examples of WPSA (2013) (Table 1). The de-hulled, solvent-extracted SBM was included at the expense of cornstarch, thus making SBM the only source of variation in P content of the diets. Analytical characterization of the SBM is presented as a footnote in Table 1. Titanium dioxide was included (0.5%) as the indigestible marker. The concentrations of total P and phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate)-P; **$InsP_6$-P**) in the diets ranged from 3.0 to 4.6 and 1.6 to 2.3 g/kg dry matter (**DM**), respectively. The Ca:total P ratio was analyzed in the diets to be 1.5:1.0, which was very close to the ratio recommended by WPSA (2013) (1.3:1.0 to 1.4:1.0). Diets were manufactured at Research Diet Services (location Wijk bij Duurstede, Netherlands). First, all ingredients except the variable ones (SBM, cornstarch, and limestone) were mixed in one lot. Then, diets A and C were mixed by adding the respective amounts of SBM, cornstarch and limestone. Finally, diet B was mixed by 50% diet A and 50% diet C. All diets were pelleted (die hole: 3.0 mm × 17 mm) with a little steam addition. Temperature of the pellets measured directly after leaving the die was 77 °C for diets A and B and 74 °C for diet C. The experimental diets were bagged, sealed, and transported to the 17 participating stations.

Table 1. Composition of the experimental diets.

Diet	A	B	C
Ingredients (g/kg)			
Soybean meal from de-hulled seed[1]	400.0	510.0	620.0
Cornstarch	448.6	336.6	224.6
Limestone (finely ground)	7.4	9.4	11.4
Soybean oil	30.0	30.0	30.0
Dried egg albumen	18.0	18.0	18.0
Sucrose	80.0	80.0	80.0
Sodium chloride	3.0	3.0	3.0
DL-Methionine	2.7	2.7	2.7
L-Threonine	0.3	0.3	0.3
Titanium dioxide	5.0	5.0	5.0
Vitamin and trace element premix[2]	5.0	5.0	5.0
Analyzed (g/kg, on dry matter basis)			
CP	231	288	339
Total P[3]	3.02 (0.06)	3.80 (0.08)	4.59 (0.08)
Ca[3]	4.57 (0.09)	5.65 (0.09)	6.78 (0.13)
Ti[3]	3.20 (0.07)	3.18 (0.07)	3.17 (0.07)
$InsP_6$-P	1.63	1.97	2.28
Ins(1,2,4,5,6)P_5-P[4]	0.24	0.27	0.32

[1]The soybean meal contained per kg of dry matter (per analyses): 541 g crude protein, 76 g ash, 36 g crude fat, 41 g crude fiber, 116 g neutral detergent fiber, 72 g acid detergent fiber, 7.1 g P, 4.3 g $InsP_6$-P, 3.3 g Ca.

[2]Premix provided the following (per kilogram of diet): vitamin A, 12.000 IU; cholecalciferol, 63 μg; vitamin E, 50 IU; vitamin K_3, 1.5 mg; vitamin B_1, 2.0 mg; vitamin B_2, 7.5 mg; vitamin B_6, 3.5 mg; vitamin B_{12}, 20 mcg; niacin, 35 mg; D-pantothenic acid, 12 mg; choline chloride, 460 mg; folic acid, 1.0 mg; biotin, 0.2 mg; Fe, 80 mg (as $FeSO_4 \cdot H_2O$); Cu, 12 mg (as $CuSO_4 \cdot 5H_2O$); Mn, 85 mg (as MnO); Zn, 60 mg (as $ZnSO_4 \cdot H_2O$); Co, 0.4 mg (as $CoSO_4 \cdot 7H_2O$); I, 0.8 mg (as KI); Se, 0.15 mg (as Na_2SeO_3).

[3]Mean (standard deviation) of analyses made for each station.

[4]All other lower inositol phosphate isomers were below the limit of quantification.

Birds and Experimental Procedures

Animal trials at the participating stations were conducted between March and September 2014. All animal

Table 2. Summary of the broiler chicken trials conducted in the collaborating stations.

Station number	Broiler strain	Sex	No. of experimental replicates per diet	Birds per replicate	Age when placed on treatment diets (d)	Age at sampling (d)	BW before sampling (kg)	ADFI (g)	Feed/gain during the experimental period	Method of killing
1	Ross 708	male	8	8	20	25	1.12	123	1.63	CO_2 asphyxiation
2	Ross PM3	unsexed	8	8	23	28	1.86	174	1.22	Pentobarbital injection
3	Ross 308	unsexed	6[1]	8	19	27	1.46	138	1.32	Cervical dislocation
4	Ross 308	unsexed	6	8	22	28	1.84	163	1.27	Cervical dislocation
5	Ross 308	male	6	10	22	27	1.03	81	1.26	CO_2 asphyxiation[2]
6	Ross 308	unsexed	6	8	23	28	1.52	151	1.06	Cervical dislocation
7	Ross 308	male	8	12	17	22	1.16	119	1.36	Cervical dislocation
8	Ross 308	male	6	12	20	27	1.57	133	1.35	Pentobarbial injection
9	Heritage 5632	male	6	8	16	21	0.82	93	1.43	CO_2 asphyxiation
10	Ross 708	unsexed	6	8	20	25	1.11	109	1.44	CO_2 asphyxiation
11	Ross 308	unsexed	7	8	18	23	1.03	93	1.42	CO_2 asphyxiation
12	Ross 308	male	8	8	16	25	1.45	98	1.32	Stunning
13	Ross PM3	unsexed	10	8	14	21	1.02	103	1.42	Cervical dislocation
14	Ross PM3	male	6[1]	8	17	24	1.30	128	1.37	Pentobarbital injection
15	Ross 308	unsexed	6	10	18	28	1.72	140	1.40	Cervical dislocation
16	Ross PM3	unsexed	10[1]	4	12	22	1.00	70	1.28	CO_2 asphyxiation
17	Ross 308	male	6	12	14	24	1.24	96	1.31	Sedanum and Ketamin

[1]Each replicate comprised two pooled cages.
[2]CO_2 asphyxiation following anesthesia with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2.

procedures were in accordance with the animal welfare regulations that were applicable for the individual participating station. While basic principles of the WPSA (2013) protocol were applied in all laboratories, details of the trials were specific for each station (Table 2). Broiler chicks (Ross 308, Ross 708, Ross PM3, and Heritage 5632) were fed commercial starter feed until they were between 12 and 23 d old when the treatment diets were introduced. At least 6 experimental replicates were used per diet by each station, and each replicate had a minimum of 8 birds. One laboratory used only 4 birds per replicate but 10 replicates per diet. Feed and drinking water were offered for ad libitum consumption. The ADFI and average BW were recorded. Birds were fed the experimental diets for a minimum period of 5 d and then were sacrificed at an age of 21 to 28 days. The abdominal cavity was immediately opened, the digestive tract removed, and the ileum (section between Meckel's diverticulum and 2 cm anterior to the ileo-ceco-colonic junction) dissected. The digesta of the distal half of the ileum was obtained by flushing with water or by gentle squeezing. Digesta from all birds of one replicate were pooled into one sample. Samples were dried (freeze drying or oven drying), ground, and sent to the Hohenheim laboratory together with the respective diet samples for chemical analyses.

Chemical Analyses

Proximate nutrients in the SBM and CP concentration in the diets were analyzed according to the official methods (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, 2007). Analysis of Ca, P, and Ti in diets and digesta samples was performed using an inductively coupled plasma optical emission spectrometer following a sulfuric and nitric acid wet digestion with all specifications described by Zeller et al. (2015b). Concentrations of $InsP_6$ and lower inositol phosphates in the diets were analyzed following EDTA extraction at pH 10 using high-performance ion chromatography as described by Zeller et al. (2015c). Digesta samples from 4 stations that had freeze-dried the samples and were found to be different in pcdP values were also analyzed for $InsP_6$ using the same method as used for the diets. This was not planned from the beginning and is not part of the WPSA (2013) protocol. Hence, some stations oven-dried the samples. Oven-dried samples were not considered for $InsP_6$ analysis because some $InsP_6$ might be hydrolyzed after sampling from the ileum during the drying process.

Calculations and Statistics

Prececal digestibility of P and Ca, and disappearance of $InsP_6$ (y) were calculated on a pen basis according to the following equation:

$$\begin{aligned} y\,(\%) &= 100 - 100 \\ &\times \left(\frac{\text{Ti in the diet (g/kg DM)}}{\text{Ti in the digesta (g/kg DM)}} \right) \\ &\times \left(\frac{\text{InsP}_6 \text{ or P or Ca in the digesta (g/kg DM)}}{\text{InsP}_6 \text{ or P or Ca in the diet (g/kg DM)}} \right) \end{aligned}$$

The amount of pcdP (g/kg DM) was calculated by multiplication of the P concentration of the diet (g/kg DM) and the respective digestibility (%) divided by 100.

Statistical evaluation of the data used the MIXED procedure of SAS for Windows (Version 9.3, SAS Institute, Cary, NC). Evaluation of P digestibility, Ca digestibility, and $InsP_6$ disappearance values of the diets was performed using diet (A, B, C), stations (1 to 16), and their interaction as fixed effects. The trial in one

station generated questionable digestibility data for P and Ca that could not be explained. Hence data of this trial were not included in the data evaluation and only 16 trials were used.

Digestibility of P from SBM was calculated for each of the 16 stations by linear regression. Linear regressions of the type y = a + mx were calculated using the SOLUTION statement to describe the relationship between pcdP content and P content in the diet (both in g/kg DM) for each station. Because differences in the P content between diets originated only from SBM inclusion, calculated slopes multiplied by 100 are regarded as an estimate of the pcdP of SBM (WPSA, 2013). The R^2 and root MS error are reported as measures for the goodness of fit. Differences in pcdP of SBM among stations were compared using the ESTIMATE statement.

RESULTS

The average pcdP of the diets across all stations for diets A, B, and C were 67, 61, and 55%, respectively (Figure 1). Effects of diet, station, and their interaction were statistically significant ($P < 0.05$). The ranges of pcdP values of the diets across stations were 55 to 82% for diet A, 46 to 79% for diet B, and 45 to 69% for diet C (Table 3).

When the pcdP concentration of the diets was regressed against total P concentration, estimated slopes of the linear regressions ranged among stations from 0.19 to 0.51 (Table 4). Even when the 2 lowest and 2 highest slopes were disregarded, the slopes of the remaining stations still ranged from 0.22 to 0.42 with significant differences among stations.

The average prececal (**pc**) disappearance of $InsP_6$ as studied in 4 stations varied from 58 to 74%, 43 to 67%, and 23 to 58% for diets A, B, and C, respectively

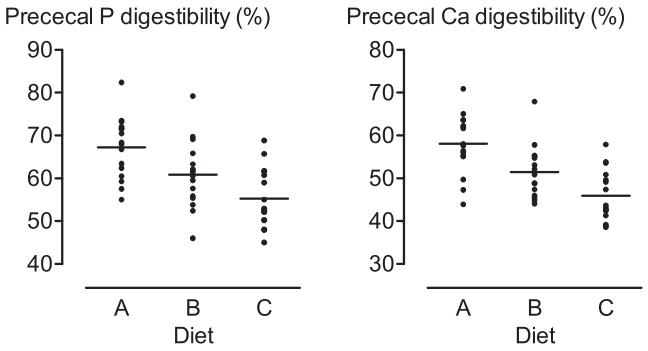

Figure 1. Scatter plot of prececal digestibility data from 16 broiler chicken trials conducted in 16 stations and using diets containing 400 (A), 525 (B), or 650 (C) g/kg soybean meal (each dot shows the mean of one station for the respective diet).

(Table 5), thus confirming the trends in differences seen in pcdP of the diets. Increments in dietary $InsP_6$ with increasing SBM level of the diet were not related to an increase of pc $InsP_6$ disappearance (Figure 2). However, across all diets and the 4 stations, a relationship between the pc disappearance of $InsP_6$-P and pcdP content became apparent, indicating that a very high proportion of the $InsP_6$-P that disappeared until the end of the ileum was absorbed (Figure 3).

The average prececal digestibility of Ca (**pcdCa**) of the diets across all stations was 57% (diet A), 51% (diet B), and 46% (diet C), with a similar range among stations as found for pcdP (Figure 1). Effects of diet, station, and their interaction on pcdCa were statistically significant ($P < 0.05$). Within the range studied the relationship between pcdP and pcdCa in the diets was linear and not significantly different among diets (Figure 4). On average, the concentration of pcdCa in the diets increased by 0.97 g/kg DM with each 1.00 g/kg DM increase in pcdP.

Table 3. Prececal digestibility of P and Ca of diets with variable inclusion of soybean meal and limestone as determined in 16 trials with broiler chickens.

		P digestibility[1] (%)						Ca digestibility[1] (%)					
Diet		A		B		C		A		B		C	
No. of station	n[2]	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	8	58	2.7	52	5.0	48	4.9	47	8.9	47	6.6	39	8.6
2	8[3]	67	3.2	58	3.3	52	1.2	64	3.4	55	5.6	47	3.3
3	6	68	4.8	62	2.2	50	3.2	56	5.0	49	3.1	42	3.4
4	6[4]	63	3.9	56	3.7	52	2.1	65	9.2	49	4.8	44	3.5
5	6	73	1.9	69	2.9	66	2.9	62	2.5	58	2.3	54	3.8
6	6	61	3.8	60	4.1	53	7.2	55	7.5	51	11.6	41	7.4
7	8	59	5.3	54	3.2	50	4.0	50	4.9	44	4.8	39	3.4
8	6	71	2.2	62	4.9	62	3.6	62	2.9	53	5.5	54	4.2
9	6	62	4.4	55	2.9	48	3.9	55	7.5	45	5.6	39	6.6
10	6	68	2.5	61	2.9	53	1.5	58	4.0	52	6.5	43	3.7
11	7	55	5.5	46	5.3	45	6.2	56	6.3	46	5.1	43	5.7
12	8	71	4.0	61	4.2	55	5.2	64	4.3	55	3.8	49	4.6
13	10	72	5.6	70	6.3	62	2.7	58	5.8	55	9.7	51	6.4
15	6	74	2.3	63	9.7	61	6.9	62	1.7	51	8.7	50	7.8
16	10	72	2.8	66	4.9	59	4.9	44	5.7	45	7.6	43	7.7
17	6	82	1.6	79	3.2	69	2.4	71	3.4	68	2.7	58	4.0

Diets: A = 400 g/kg soybean meal; B = 510 g/kg soybean meal; C = 620 g/kg soybean meal.
[1]Effects of diet, station, and their interaction were statistically significant ($P < 0.05$).
[2]Number of replicates (cages) per diet. [3]n = 7 for diet C. [4]n = 5 for diets A and C.

Table 4. Results of linear regression analysis of prececal digestible P concentration (y, g/kg DM) in function of dietary P concentration (x, g/kg DM) using diets with incremental inclusion levels of soybean meal.

No. of station	Intercept	*SE*	Slope	*SE*	R^2	Root MS error
1	0.83	*0.30*	$0.31^{a,b,c}$	*0.07*	>0.99	0.01
2	1.37	*0.41*	$0.22^{b,c}$	*0.08*	0.97	0.02
3	1.50	*0.43*	0.19^{c}	*0.07*	0.68	0.09
4	0.99	*0.40*	$0.30^{a,b,c}$	*0.07*	0.98	0.03
5	0.66	*0.42*	0.51^{a}	*0.08*	>0.99	<0.01
6	0.72	*0.42*	$0.38^{a,b,c}$	*0.08*	0.93	0.07
7	0.79	*0.42*	$0.33^{a,b,c}$	*0.08*	>0.99	<0.01
8	0.80	*0.42*	$0.43^{a,b}$	*0.07*	0.95	0.06
9	1.34	*0.42*	0.19^{c}	*0.08*	0.91	0.04
10	1.36	*0.43*	$0.25^{b,c}$	*0.07*	0.94	0.04
11	0.81	*0.42*	$0.27^{b,c}$	*0.08*	0.92	0.05
12	1.34	*0.42*	$0.26^{b,c}$	*0.07*	>0.99	0.02
13	0.94	*0.42*	$0.42^{a,b}$	*0.07*	0.95	0.07
15	1.02	*0.41*	$0.38^{a,b,c}$	*0.07*	0.97	0.04
16	1.12	*0.41*	$0.34^{a,b,c}$	*0.08*	>0.99	0.02
17	1.37	*0.42*	$0.41^{a,b,c}$	*0.08*	0.91	0.08

[a-c]Superscript letters indicate significant differences between slopes.

Table 5. Prececal disappearance of $InsP_6$ of diets with variable inclusion of soybean meal as determined in 4 trials with broiler chicken.

Diet		Prececal $InsP_6$ disappearance[1] (%)					
		A		B		C	
No. of station	n[2]	Mean	SD	Mean	SD	Mean	SD
4	6[3]	70	4.5	52	8.5	47	5.0
5	6	74	3.8	67	4.7	58	7.1
7	8	58	10.8	47	8.1	36	8.9
9	6	62	4.3	43	3.6	23	11.4

Diets: A = 400 g/kg soybean meal; B = 510 g/kg soybean meal; C = 620 g/kg soybean meal.

[1]Effects of diet, station, and their interaction were statistically significant ($P < 0.05$).

[2]Number of replicate cages.

[3]n = 5 for diets A and C.

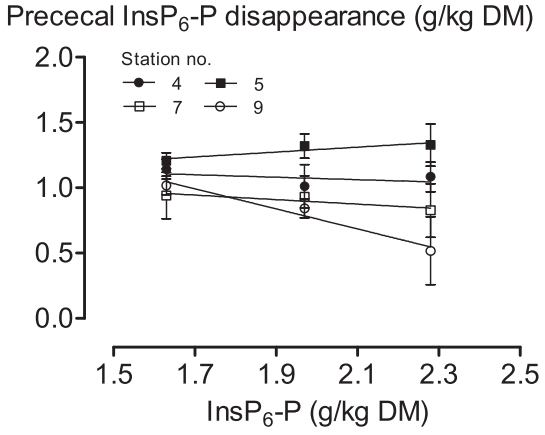

Figure 2. Relationship between the concentration of $InsP_6$-P in the diet and prececal $InsP_6$-P disappearance in diets with different levels of soybean meal and studied in 4 broiler trials in 4 stations (means and SD). Slopes estimated for stations 4, 5, and 7 did not deviate from zero ($P > 0.05$).

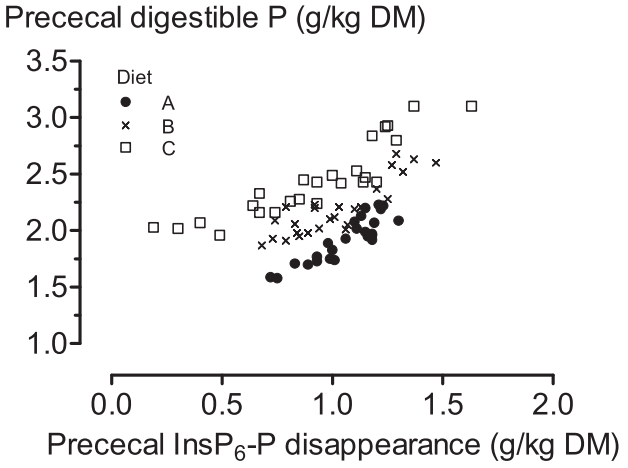

Figure 3. Relationship between the concentration of prececal digestible P in dependence of prececal $InsP_6$-P disappearance in diets including different levels of soybean meal and studied in 4 broiler trials in 4 stations (each symbol shows the value of one cage). The slopes of the linear regressions calculated for each diet were not significantly different ($P = 0.27$), and the pooled slope was 0.91.

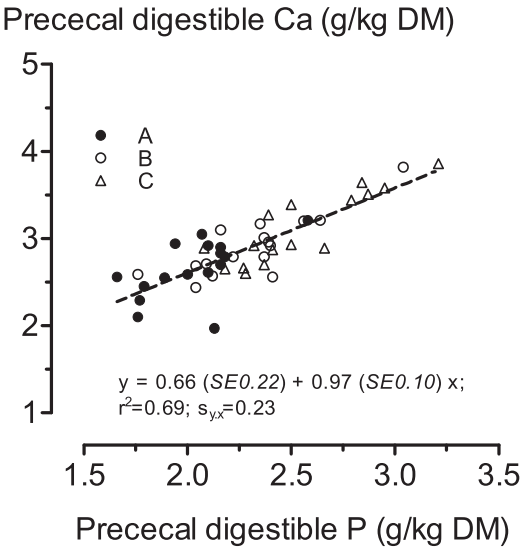

Figure 4. Relationship between the concentration of prececal digestible P and prececal digestible Ca in diets fed to broilers and using diets containing 400 (A), 510 (B), or 620 (C) g/kg soybean meal (each symbol shows the mean of one out of 16 stations for the respective diet). The slopes determined separately for each diet were not significantly different.

DISCUSSION

The underlying hypothesis of this study was that digestibility values determined for the diets and for the SBM are similar among stations. This hypothesis must be rejected on the basis of the results. The pcdP for the same SBM ranged between 19 and 51% among stations, which can be a frustrating fact from the viewpoint of feed producers aiming to use P digestibility data in their feed formulation matrix. It is of note that the opposite was found in a ring test on pcd of amino acids in broilers (V. Ravindran et al., personal communication).

There, pcd of amino acids of a corn-SBM-based diet was similar among the 5 collaborative stations, once important protocol details had been agreed on. Probably there is something specific on the targeted nutrient (amino acids vs. P and $InsP_6$) that caused high variation in one ring test but not in the other. It cannot be ruled out that this difference is related to hormonal control of absorption known for Ca and P, but not for amino acids. However, for reasons subsequently mentioned, other factors are seen more likely involved.

In the current ring test we used the same diets in all stations to mitigate variation potentially caused by raw material origin or diet processing. All diet and digesta samples were analyzed in the same laboratory, hence minimizing variation of results potentially caused by differences in analytical protocols. In spite of this standardization, large differences occurred. This variation is most likely associated with how the experiment was conducted and birds managed in each station. While most assay details are standardized in the applied protocol (WPSA, 2013), some were not and will be discussed herein.

The starter diets used in the pre-experimental period met the bird's requirements, but were specific for each station and not of the same ingredient composition. Digestive capacity adaptation to Ca and P deficiencies or imbalances can ocurr within 48 h (Angel et al., 2013). Hence, any adaptation to the experimental diets would have already occured in the present study before samples were taken. However, it cannot be ruled out that starter diet details have affected the measurements subsequently made with the experimental diets. The P concentration of the starter diets (formulated values) was between 0.5 and 0.7%, and the Ca concentration between 0.7 and 1.1%. Some of the starter diets contained an added phytase while others did not. Compensatory adaptation in growth and bone mineralization in a later growth phase occurred when broilers were fed a diet moderately deficient in P and Ca from hatching to 18 d (Yan et al., 2005). A recent study showed that a low level of P in the diet fed from 10 to 21 d can affect the mRNA levels of several genes encoding Ca and P transporters at 35 d of age, depending on the amount of P and Ca fed from 22 to 35 d (Rousseau et al., 2016). These authors concluded that chickens are able to adapt to early dietary changes in P and Ca through improvement of digestive efficiency in a later phase. Starter diet P and Ca levels were different across participating stations in the current study, but none was deficient. It cannot be ruled out but is not substantiated with the data that the differences affected the results. A negative trend might be interpreted when the P digestibility data are related to the P level of the starter diets (Figure 5A); however, the regression line did not significantly deviate from zero ($P = 0.08$). A relationship with the formulated phytase content of the starter diet also was not apparent (Figure 5B). Because only the formulated values of P, Ca, and phytase are available for the starter diets, and because we do not have information about raw material composition of the starter diets, relationships between starter diet characteristics and determined digestibility values should be viewed with caution. In such complex design, colinearity also among other factors of variation might occur (starter diet, duration of period, age, etc.) and thus could bias interpretation.

Some starter diets contained a coccidiostat, while others did not. As a consequence, microbiota colonization of the digestive tract might have developed differently among stations, which could affect $InsP_6$ hydrolysis as seen in the differences among stations and diets (Table 5). Previous studies have found a relatively high level of prececal or excreta $InsP_6$ disappearance in birds (25 to 78%) even when diets devoid of any supplemental or plant-intrinsic phytase were used (Applegate et al., 2003; Tamim et al., 2004; Delezie et al., 2012; Amerah et al., 2014; Zeller et al., 2015a; Zeller et al., 2015b; Li et al., 2016). This range of values is high and probably related to dietary Ca levels or level and origin of $InsP_6$. However, the generally high $InsP_6$ disappearance values strongly indicate that enzymes of non-feed origin, namely, endogenous mucosal and bacterial phytases, have caused a substantial $InsP_6$ hydrolysis. Little is known so far about the contribution of specific bacteria to phytase activity in the digestive tract of chickens. However, lactobacilli were shown to be the main colonizers in the crop, jejunum, and ileum of broilers (Witzig et al., 2015). Some of the lactobacilli strains that were detected in gut content of broilers have been characterized to express high phytase activity (Palacios et al., 2008; Taheri et al., 2009). Use of the coccidiostat monensin reduced abundance of *Lactobacillus* sp. in ceca content (Danzeisen et al., 2011) and nisin-sensitive *L. reuteri* isolates were detected in the crop (Abbas Hilmi et al., 2007). Hence it is possible that coccidiostats included in some but not all starter diets used in the current ring test reduced abundance of bacteria known to possess phytase activity and thus $InsP_6$ hydrolysis. Initial microbiota colonization in the starter phase probably was effective in the experimental phase and contributed to $InsP_6$ hydrolysis from SBM to a different extent. We observed a very close relationship between $InsP_6$-P disappearance and pcdP content of the diets (Figure 3), and although P of non-$InsP_6$ origin in SBM also contributed to the change in pcdP content, the relationship well demonstrated that $InsP_6$ hydrolysis was a major determinant for the pcdP of SBM.

Another factor of potential relevance for microbial colonization and development of the digestive tract is the way birds were kept in the pre-experimental period. When birds were raised in floor pens, litter material intake could affect digestive tract development in general and microbial colonization in particular. Floor pen raising was practiced in some of the stations because it was required as per approval from the ethics committee or for other reasons.

Endogenous enzymes involved in intestinal $InsP_6$ hydrolysis also can originate from epithelial secretion because studies using purified brush border membrane vesicles from different sections of the small intestine of

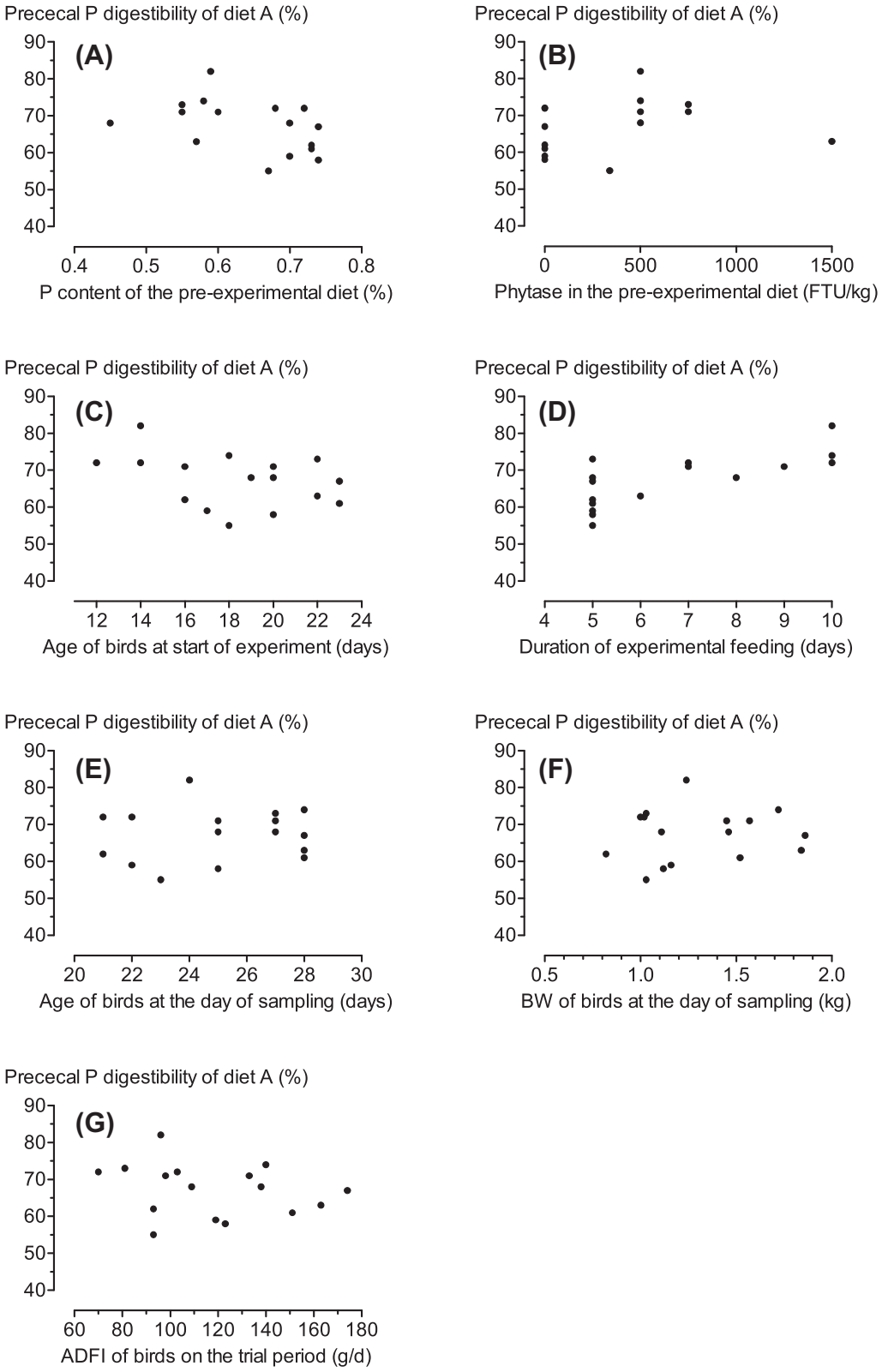

Figure 5. Comparison between the prececal digestibility of P in diet A with the formulated P concentration of the pre-experimental starter feed (panel A), the supplemented phytase of the pre-experimental starter feed (panel B), the age of birds when the experimental diets were introduced (panel C), the duration of the experimental feeding period (panel D), the age of birds at the d of sampling (panel E), the BW of the birds at the d of sampling (panel F), and the ADFI in the trial period (panel G). Each dot shows the mean of one station. The difference to zero of the calculated slopes of linear regressions was significant in panel D ($P < 0.01$), but not in panel A ($P = 0.08$) or any other panel ($P > 0.1$).

broiler chickens reported phytase activity (Maenz and Classen, 1998; Onyango et al., 2006; Huber et al., 2015). Significant differences in the V_{max} of epithelial phytase kinetics were found between Hubbard × Peterson and Ross × Ross broilers, but not in pc $InsP_6$ hydrolysis (Applegate et al., 2003). It has been hypothesized from genetic studies that epithelial phytase expression is affected by the bird's genetic background (Beck et al., 2014). Indeed, genomic studies showed significant heritability in the range of 0.10 to 0.22 for P utilization, P excretion rate, and phytate-P bioavailability in broilers and Japanese quail (Zhang et al., 2003; de Verdal et al., 2011; Beck et al., 2016). In the current ring test, fast growing broilers from different strains were used that were typical for the respective station. It is possible that the genetic background of the parent lines used for broiler production in different regions of the world was different and this contributed to the differences we found among stations. However, it is not currently possible to quantify the contribution of epithelial phytase to intestinal luminal $InsP_6$ hydrolysis.

Some of the stations involved in the current study used unsexed broilers while others used males. As a side aspect of the current study one station has compared males and females when studying digestibility of the experimental diets (Schedle et al., 2016). The authors reported almost identical values and no significant differences between males and females. This let us conclude that for the current study it was not relevant whether mixed-sex or male broilers were used.

Some other characteristics of the experiments such as growth, ADFI, age at sampling, and killing procedures were also different among stations. Statistical search for any relationship would not be meaningful because the study was not designed to investigate any of the effects and the data set is too limited for correlation analysis. However, it does not appear from regressions that age at slaughter or at start of experimental feeding, BW at slaughter, or ADFI in the trial period affected the results (Figure 5).

The protocol of WPSA (2013) has standardized several details for the determination of P digestibility. From the results of this ring test, we conclude that the protocol standardization must go beyond the standards already set. Differences in starter diet composition and management conditions during the pre-experimental period of the trials affected the results and thus also need standardization. Until a more standardized protocol is established, care must be exercised when comparing P digestibility data from different laboratories.

ACKNOWLEDGMENTS

The financial support for this project provided by BASF SE, Nutreco, and Adisseo is gratefully acknowledged. Authors are also grateful to M. Schollenberger, M. Liebscher, and H. Ott for conducting chemical analyses.

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