

# Relative phytase efficacy values as affected by response traits, including ileal microbiota composition

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**ABSTRACT** The objective of this study was to compare the effects of graded inclusions of 2 phytase products and a mineral P source in broiler chickens using different response traits, including ileum microbiota composition. Eleven experimental diets were used. These were a low-P basal diet and diets supplemented with increasing levels of dicalcium phosphate (**DCP**), Natuphos E 5000 G (**NE**), or Natuphos 5000 G (**N**). The performance traits, prececal P digestibility, and tibia and foot ash results were subjected to regression analysis and slope ratios were used to compare the supplements based on the measured evaluation traits. In the microbiota analysis, total nucleic acids were extracted and the 16S rRNA gene was targeted for use in the amplicon sequencing process. Phylogenetic analysis was performed using Mothur, followed by a multivariate statistical analysis. The various

response traits caused different estimates of relative efficacy. The mean results of all the response traits showed that a 1.75-fold increase in the activity of N was needed to achieve the same response as NE and the variability among the detected traits ranged from 1.59 (prececal digestible P intake) to 1.91 (amount of tibia ash). The mean slope ratio between DCP and NE was 311 and varied between 208 (ADG) and 349 (foot ash concentration). The mean slope ratio for phytase N with DCP was 552 and varied from 357 (ADG) to 640 (tibia ash concentration). The ileum microbiota composition was not different among the diets. A similar composition was driven in the abundance of *Lactobacillus crispatus*, *Lactobacillus salivarius*, and *Lactobacillus gallinarum*. The results suggest that different response traits cause markedly different estimates of relative phytase efficacy.

**Key words:** evaluation, method, digestibility, bone, microbiota

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## INTRODUCTION

Different phytase products are used by the broiler industry. These phytases degrade *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) (**InsP<sub>6</sub>**) and its salts (phytate), which are the major forms of P in plant feedstuffs. Phytate is only partially degraded by endogenous enzymes in the animal. Therefore, phytase supplements are currently being used to increase P utilization by the animal. Different phytase products have specific activity characteristics, such as an optimum pH or resistance to proteolysis, and thus may have different InsP<sub>6</sub> degradation efficiencies in the gastrointestinal tract. Depending on the genetic background, phytases

start dephosphorylation of InsP<sub>6</sub> at a specific position on the inositol ring and follow a certain degradation cascade (Konietzny and Greiner, 2002). Two major groups are 6- and 3-phytases. The number indicates that dephosphorylation is initiated at the sixth and third phosphate group on the inositol ring, respectively.

There are various approaches currently used to determine available P in broilers. Prececal digestible P (**pcdP**) is the most established trait for quantitative purposes (WPSA, 2013), while bone-related data, BW gain, and blood inorganic P concentration are used to detect the relative P bioavailability (Shastak and Rodehutschord, 2013). The most often used bone for this kind of analysis is the tibia. However, other bones, such as the femur, toes, or the complete foot, are also described as response traits (Yan et al., 2005; Malloy et al., 2017). Shastak et al. (2012a) compared different bone measurements, pcdP, P retention, and blood inorganic phosphate in response to different mineral P supplements. They concluded that bone criteria and P retention or

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pcdP led to different rankings for the values of the 2 mineral P sources tested. Hence, there may be differences in the relative values for different phytase products depending on the response trait that is measured.

To some extent, phytase is endogenously provided by the epithelial tissue of broilers and by some of the microorganisms in the gastrointestinal tract. *Lactobacillus* species, such as *L. salivarius*, *L. plantarum*, and *L. pentosus*, are able to produce enzymes with phytase-like activity (Lee et al., 2013; Sumengen et al., 2013; Amritha et al., 2017). Microorganisms in the gastrointestinal tract may contribute to the degradation of phytate and other fractions in the feed. The abundance of microbial groups can be influenced by feedstuffs and supplements, such as phytase or mineral P (Witzig et al., 2015; Borda-Molina et al., 2016). However, as far as can be ascertained, the effects of different phytase products on intestinal microbiota composition have not yet been compared.

The objective of this study was to compare 2 phytase products and dicalcium phosphate (DCP) using several response traits, including ileal microbiota composition. Each of the 3 supplements was fed at different inclusion levels, which meant that the results could be analyzed using regression analysis and slope ratios. The hypotheses were that the relative efficacy values of phytase products and DCP depend on the chosen response trait and that ileal microbiota composition responses differ among products.

## MATERIALS AND METHODS

### Birds and Housing

The trial was conducted at the Agricultural Research Station of the University of Hohenheim, Germany. All procedures were performed in accordance with the German Animal Welfare Legislation requirements and

approved by the Regierungspräsidium Tübingen, Germany (project no. HOH 37/15 TE). A total of 792 unsexed Ross 308 broiler hatchlings were supplied by a commercial hatchery (Brütereier Süd GmbH & Co. KG, Regenstauf, Germany) and allocated to 66 pens (115 × 230 cm ground area, 260 cm height), which were stocked with 12 hatchlings each. On d 7, 6 pens were assigned to each of the 11 treatments according to a randomized complete block design. The birds were kept on wood shavings bedding until d 14, and on perforated floors from d 14 to d 22. Feed and tap water were provided for *ad libitum* consumption during the trial. The lighting program was 24 L:0 D until d 3, 22 L:2 D until d 7, 16 L:8 D until d 10, and 12 L:12 D until the end of the experiment. The temperature was set at 34°C on the day of placement and continuously decreased to 24°C on d 22. The well-being of the birds was checked at least twice daily.

### Diets and Treatments

The birds received a commercial pelleted starter feed in the pre-experimental phase until d 7 (4150020 Club Kükenmaststarter, Deutsche Tiernahrung Cremer GmbH & Co. KG, Düsseldorf, Germany). During the experimental phase (d 7 to 22), the birds were fed corn and solvent-extracted soybean meal-based pelleted diets (Table 1). The basal diet (BD) contained 4.5 g P/kg DM and 7.9 g Ca/kg DM but no mineral P or exogenous phytase. The other diets had a similar ingredient composition but contained increasing amounts of 1 of 3 supplements: DCP (0.7, 1.4, 2.1, and 2.9 g P/kg from DCP), NATUPHOS E 5000 G (NE; 250, 500, and 750 FTU/kg), or NATUPHOS 5000 G (N; 250, 500, and 1000 FTU/kg). The enzymes were supplied by BASF SE (Lampertheim, Germany). When DCP was added, the inclusion of calcium carbonate was adjusted to maintain

**Table 1.** Ingredient composition and analyzed concentrations of the experimental diets.<sup>1</sup>

Ingredient, g/kg	BD	DCP 0.7	DCP 1.4	DCP 2.1	DCP 2.9	NE250	NE500	NE750	N250	N500	N1000
Corn	575	575	575	575	575	575	575	575	575	575	575
Soybean meal	335	335	335	335	335	335	335	335	335	335	335
Rapeseed meal	30	30	30	30	30	30	30	30	30	30	30
Soybean oil	15	15	15	15	15	15	15	15	15	15	15
Calcium carbonate	14	14.5	15	15.5	16	14	14	14	14	14	14
Diamol <sup>2</sup>	15	11.3	7.5	3.8	-	15	15	15	15	15	15
Dicalcium phosphate	-	3.2	6.5	9.7	13	-	-	-	-	-	-
Vitamin/mineral mix <sup>3</sup>	5	5	5	5	5	5	5	5	5	5	5
TiO <sub>2</sub>	5	5	5	5	5	5	5	5	5	5	5
Sodium bicarbonate	3	3	3	3	3	3	3	3	3	3	3
DL-methionine	2	2	2	2	2	2	2	2	2	2	2
Sodium chloride	1	1	1	1	1	1	1	1	1	1	1
Phytase (FTU/kg)	0	0	0	0	0	250	500	750	250	500	1000
Analysed concentrations											
Ca (g/kg DM)	7.9	9.0	10.3	11.4	12.7	7.8	8.1	7.9	8.0	8.0	7.9
P (g/kg DM)	4.5	5.2	5.9	6.6	7.4	4.5	4.5	4.5	4.5	4.5	4.5
InsP <sub>6</sub> -P (g/kg DM)	2.9	2.9	2.9	2.8	2.9	2.9	2.9	2.9	2.9	2.9	3.0
Phytase (FTU/kg)	< 60	< 60	< 60	< 60	< 60	240	580	730	330	600	1130

<sup>1</sup>BD: basal diet, DCP: dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G.

<sup>2</sup>Purified diatomaceous earth mainly consisting of SiO<sub>2</sub>.

<sup>3</sup>Provided per kilogram of complete feed: vitamin A (retinyl acetate): 12,000 IU; vitamin D<sub>3</sub> (cholecalciferol): 2,500 IU; vitamin E (dl- $\alpha$ -tocopherol): 50 mg; vitamin K<sub>3</sub> (menadione): 1.5 mg; vitamin B<sub>1</sub> (thiamine): 2.0 mg; vitamin B<sub>2</sub> (riboflavine): 7.5 mg; vitamin B<sub>6</sub> (pyridoxine): 3.5 mg; vitamin B<sub>12</sub> (cyanocobalamin): 20  $\mu$ g; niacin: 35 mg; pantothenic acid: 12 mg; choline chloride: 460 mg; folic acid: 1.0 mg; biotin: 0.2 mg; iron: 80 mg; copper: 12 mg; manganese: 85 mg; zinc: 60 mg; iodine: 0.8 mg; selenium: 0.15 mg; anti-oxidant: 125 mg.

a constant P:Ca ratio. Both DCP and calcium carbonate were supplemented at the expense of diamol. The phytase products were additions to the general mixture. All experimental diets contained 5 g/kg TiO<sub>2</sub> as an indigestible marker and were pelleted through a 2.5-mm die. Intended concentrations of P, Ca, and phytase were confirmed by analysis (Table 1). The feed was produced by “Research Diet Services” (Hoge Maat 10, 3961 NC Wijk bij Duurstede, Netherlands).

### Sampling and Measurements

The birds and feed were weighed on a pen basis before placement on d 7 and before slaughter to calculate the ADG, ADFI, and gain per feed ratio (G:F). Subsequently, the birds were stunned with a gas mixture of 35% CO<sub>2</sub>, 35% N<sub>2</sub>, and 30% O<sub>2</sub>, and then euthanized by CO<sub>2</sub> asphyxiation on days 21 and 22 (3 of the 6 pens from each treatment on each d). The intestinal fill was standardized by removing the feed at 2 h before slaughter, followed by 1 h *ad libitum* access to the feed. Individual BW was recorded immediately after slaughter and all the birds in a pen were sorted by weight. The right tibiotarsus (**tibia**) and foot from bird numbers 2, 5, 8, and 11 (in increasing order of BW) were excised, labeled for individual identification, and frozen at -20°C. The small intestine was excised from all the birds in each pen and the posterior half of the section between Meckel’s diverticulum and 2 cm anterior to the ileo-caeco-colonic junction was identified. A small piece that was approximately 2 cm from the anterior end of this section of each bird was cut lengthwise. The digesta was gently removed with a spatula without scraping the mucosa, pooled on a pen basis, mixed, and stored on ice until it was frozen at -80°C for microbiota analysis. The remaining part of the ileum section was flushed with cold deionized water, the content pooled on a pen basis, and immediately frozen at -20°C until it was freeze-dried. The freeze-dried samples were stored in airtight containers for further analysis.

### Chemical Analyses

Samples of the diets and freeze-dried digesta were ground in a vibrating cup mill (PULVERISETTE 9, Fritsch GmbH, Idar-Oberstein, Germany) to obtain a fine powder. The pulverized feed and digesta samples were analyzed for P, Ca, and TiO<sub>2</sub> using inductively coupled plasma optical emission spectrometry (Shastak et al., 2012b) following sulfuric and nitric acid wet digestion as described by Boguhn et al. (2009). The method of Zeller et al. (2015) was used to determine the InsP<sub>3-6</sub> isomers in the feed and digesta with slight modifications as described by Sommerfeld et al. (2018). The isomers were measured by high-performance ion chromatography (ICS-3000 system, Dionex, Idstein, Germany). It was not possible to separate the enantiomers using this methodology. Therefore, the results do not distinguish between the D- and L-forms. Some InsP<sub>3</sub>

isomers could not be identified because standards were unavailable. The term InsP<sub>3x</sub> is used for the isomers Ins(1,2,6)P<sub>3</sub>, Ins(1,4,5)P<sub>3</sub>, and Ins(2,4,5)P<sub>3</sub> with unknown proportions because clear discrimination was not possible due to co-elution. Enzyme activities were determined by BASF SE (Ludwigshafen, Germany) using ISO EN 30024 (International Organization for Standardization, 2009).

After defrosting, the adhering tissues, cartilage caps, and fibula bones were removed from the tibiae by hand and with tap water. The feet were used including skin, claws, and all tissues below the *articulatio intertarsalis*. Subsequently, the tibia bones were pre-dried at 30°C and the feet at 60°C for 48 h in a compartment oven (VL 115, VWR International GmbH, Darmstadt, Germany). Dry matter content was determined at 103°C for 48 h (tibiae) or 72 h (feet). The ash content was determined after ignition at 600°C in a muffle furnace (L 40/11/B170, Nabertherm GmbH, Lilienthal, Germany) for 24 h (tibiae) and 48 h (feet). The bones and feet were placed in the furnace at the beginning of the 7 h heat-up period and remained in the furnace for 5 h after it.

### DNA Extraction and Illumina Amplicon Sequencing

The DNA was extracted from ileal digesta samples using a FastDNA SPIN Kit for soil from MP Biomedicals (Solon, OH) according to the manufacturer’s instructions. The DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and stored at -20°C. Illumina library preparation was performed according to Kaewtapee et al. (2017). In brief, the V1-2 region of the 16S rRNA bacterial gene was amplified in a 20 µL reaction. Then, 1 µL of this first PCR product was used as a template in a second PCR using multiplexing and indexing primers as described previously (Camarinha-Silva et al., 2014). The amplicons were verified by agarose gel electrophoresis, purified, and normalized using a SequelPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA). The samples were pooled and sequenced using 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform. Mothur (v.1.40) was used to perform the bioinformatic pipeline (Kozich et al., 2013). Raw forward and reverse reads were assembled using the `make.contigs` function. Reads with ambiguous bases, more than 8 homopolymers and sequence length higher than 355 bp and lower than 250 bp were discarded using the `function screen.seqs`. Sequences were aligned against the Silva database version123 and further checked for chimeras using the `function chimera.uchime`. Reads were classified (`classify.otus`) using the `trainset14` of RDP with a cutoff of 80. An average of 73,260 ± 2942 was obtained per sample. In order to cluster sequences into operational taxonomic units (OTU) the `function cluster.split` (`splitmethod=classify`, `taxlevel=4`, `cutoff=0.03`) was used. The `function make.shared` (`cutoff=0.03`) was then used to create a table with the information of

number of reads present on each OTU and group. The consensus taxonomy of each OTU was obtained with the function `classify.otu` (label=0.03). The function `get.oturep` (label=0.03) was used to get the fasta sequence of each OTU representative. All singletons were discarded, and samples were standardized by total. Only OTUs with an average abundance higher than 0.0001% were considered for further analysis. A total of 1093 OTUs were obtained. The closest representative was manually identified using RDP's sequence match function (version 3) (Wang et al., 2007) with the following parameters (Strain: Both; Source: Both; Size: ≥1200, Quality: Good; Taxonomy: Nomenclatural; KNN matches=1). The output taxonomy table followed the defined confidence threshold cut-off value for each taxonomic level of Yarza et al. 2014: genus (94.5%), family (86.5%), order (82.0%), class (78.5%) and phylum (75.0%). A species name was given if >97% similarity was observed with the closest representative sequence. Sequences were submitted to the European Nucleotide Archive under the accession number PRJEB38381.

## Calculations and Statistical Analyses

The weight of the birds and feed consumption were recorded on a pen basis. Then, the data were used to calculate ADG, ADFI, and G:F with a correction for mortality. The prececal digestibility of P and Ca, and InsP<sub>6</sub> disappearance ( $y_{ij}$ ) were calculated using the following equation:

$$y_{ij}(\%) = 100 - 100 \cdot \left( \frac{Ti \text{ in feed}_{ij}}{Ti \text{ in digesta}_{ij}} \cdot \frac{y \text{ in digesta}_{ij}}{y \text{ in feed}_{ij}} \right),$$

where  $y_{ij}$  is the concentration of the respective analyte in the  $i_j^{\text{th}}$  sample and  $Ti$  is the titanium concentration, both in grams per kilogram DM.

A mixed model was fitted to the performance data, P and Ca prececal digestibility, InsP<sub>6</sub> disappearance, InsP isomers, tibia, and foot ash content using the MIXED procedure in SAS for Windows (version 9.4; SAS Institute Inc., Cary, NC). The following model was chosen for all traits measured per pen:

$$y_{ij} = \mu + \alpha_i + b_j + \varepsilon_{ij},$$

where  $y_{ij}$  is the observation of the response variable,  $\mu$  is the intercept,  $\alpha_i$  represents the effect of the  $i$ -th dietary treatment,  $b_j$  is the block effect of the  $j$ -th localization within the barn, and  $\varepsilon_{ij}$  is the residual error effect of  $y_{ij}$ . Treatment effects were considered as fixed effects, whereas the localization effects within the barn ( $b_j$ ) were random effects. Ash data measurements were also taken from each bird. Thus, the model was extended by adding a random pen effect ( $p_{ij}$ ) as measures were taken per animal, which was in contrast to the pen data for all other traits. The model can be shown as

$$y_{ijk} = \mu + \alpha_i + b_j + p_{ij} + \varepsilon_{ijk},$$

where  $p_{ij}$  is the random effect of pen  $i$  in location  $j$  within the barn and  $\varepsilon_{ijk}$  is the error of observation  $y_{ijk}$  from the  $k^{\text{th}}$  animal within the  $i_j^{\text{th}}$  pen. The heterogeneity of error variances between treatments were tested for each trait and the model with the smallest Akaike information criterion (Wolfinger, 1993) was used. A graphical check of the residuals for the normal distribution and homogeneity of variance (besides the heterogeneity accounted for by the model) was performed. Significant treatment effects were detected using a global F-test ( $P \leq 0.05$ ). When the F-test identified treatment differences, a multiple t-test (Fisher's LSD test) was used to explore the treatments that were different.

Additionally, the treatments were factorized into supplements and their concentrations. Concentration was used as a metric variable and a regression-type model was fitted with slopes for each supplement. The latter allowed the equivalence ratios among DCP and the 2 phytases to be calculated. These ratios were calculated using linear and nonlinear regressions, where the response of each pen was plotted against the level of phytase supplementation or P originating from DCP in the experimental diets. Linearity was tested using diet-specific means as lack of fit ( $\alpha = 0.05$ ). The MIXED and NLMIXED procedures in SAS were used to calculate the regressions. Regressions for the effects of the 3 supplements were simultaneously calculated. A common intercept was fitted for BD, NE, N, and DCP and the linear regression model used was

$$y_{ijs} = \mu + \beta_{NE}c_{NEij} + \beta_Nc_{Nij} + \beta_{DCP}c_{DCPij} + b_j + e_{ij,s}$$

where  $\mu$  represents the common intercept,  $\beta_{NE}$ ,  $\beta_N$ , and  $\beta_{DCP}$  represent the 3 slopes for supplements NE, N, and DCP, respectively, and  $c_{NEij}$ ,  $c_{Nij}$ , and  $c_{DCPij}$  are the concentrations of each supplement in the  $i_j^{\text{th}}$  pen, respectively. When there were no significant differences between the slopes, then the model could be simplified to

$$y_{ij} = \mu + \beta c_{ij} + b_j + e_{ij},$$

where  $\beta$  and  $c_{ij}$  are the common slope and the concentration used in the  $i_j^{\text{th}}$  pen, respectively. In both models, all other effects are defined as being analogous to previous models. For nonlinear regression, the following model was fitted:

$$y_{ij} = a_s + (\mu - a_s) \cdot [1 - e^{-\beta_{NE} \cdot c_{NEij}} - e^{-\beta_N \cdot c_{Nij}} - e^{-\beta_{DCP} \cdot c_{DCPij}}] + b_j + e_{ij},$$

where  $\mu$  represents the common intercept,  $a_s$  represents the  $s^{\text{th}}$  plateau values for the 3 supplements NE, N, DCP, and all other parameters are defined as being analogous to previous models. The ratios of parameters  $\beta_{NE}$ ,  $\beta_N$ , and  $\beta_{DCP}$  within each trait were calculated from estimates. When the regression was exponential, then the ratio directly represents the ratio due to the exponential function involved.

The block was taken as a random effect for the linear regression and as a fixed effect for the exponential regression. The models with random and fixed block effects are equivalent because the blocks were complete

(Möhring et al., 2015). The heterogeneity of error variances between supplements were tested as described for the mixed model.

Illumina amplicon sequencing data from the total microbial communities were analyzed using PRIMER (version 7.0.9, PRIMER-E, Plymouth Marine Laboratory, Plymouth, UK) (Clarke and Warwick, 2001) and a sample similarity matrix was created using the Bray-Curtis coefficient (Bray and Curtis, 1957). Similarity percentage analysis was used to calculate the average similarity within experimental treatments and to identify the genera principally responsible for the differences among treatments (Clarke and Warwick, 2001) and the PERMANOVA routine was used to study the significant differences obtained when the dietary treatments were studied using a permutation method under a reduced model followed by pairwise multiple comparison with Benjamini-Hochberg corrections in R (R core Team, 2017). Correlations were estimated using the Pearson correlation coefficient by PRISM 6 (GraphPad Software, San Diego, CA). A correlation was considered significant when  $P \leq 0.05$ .

## RESULTS

### Performance Traits

The average BW of the broiler chickens at the beginning of the experimental period (d 7) was 181 g and did not differ among treatments ( $P = 0.998$ , Table 2). Supplementation with DCP, NE, or N increased final BW, ADG, ADFI, and G:F in a dose-dependent manner ( $P < 0.001$ ). The largest DCP supplementation level produced the highest values for final BW, ADG, and ADFI. The G:F was greatest at the highest supplementation levels for the 3 supplements, but there were no significant differences among the supplements. A total of 41 birds (6.8%) died during the experimental period. Most of these birds were allocated to BD (19 birds), the lowest DCP supplement level (5 birds), NE (4 birds), and N (7 birds). The remaining birds were distributed among the other treatments with a maximum of 2 dead birds per treatment.

### Digestibility of P and Ca, $\text{InsP}_6$ Disappearance, and Bone Ash

Prececal P digestibility and  $\text{InsP}_6$  disappearance decreased with DCP supplementation and increased

with NE and N supplementation ( $P < 0.001$ , Table 3). The values for NE 250, N 250, and N 500 did not significantly differ from the BD values. The highest values for pcdP and  $\text{InsP}_6$  disappearance were observed when the supplementation levels of the 2 phytase products were greatest, whereas the lowest value was detected at the highest supplementation level for DCP. Prececal digested P increased as the supplement levels rose. Calcium digestibility was highest in BD and with lowest N supplementation. It decreased when the feed was supplemented with DCP, NE, and N. The lowest value occurred when DCP supplementation was highest ( $P < 0.001$ ).

The pattern for  $\text{InsP}$  isomers in the ileum digesta was similar for the 2 phytase products (Table S1). Only  $\text{Ins}(1,2,3,4)\text{P}_4$  and  $\text{Ins}(1,2,3,4,5)\text{P}_5$  were slightly higher in NE than in N, whereas it was the opposite for  $\text{Ins}(1,2,4,5,6)\text{P}_5$ . The  $\text{InsP}_6$  concentration in ileal digesta was highest in DCP 2.9, and lowest in NE 750 and N 1000. The tibia and foot ash masses were lowest in the BD group and increased as the DCP, NE, and N levels rose ( $P < 0.001$ ) (Table 3). The largest amount of ash was observed when the DCP supplementation level was highest. The largest NE and N supplementation levels produced the second highest ash mass values, but there was no significant difference between the phytase products. The ash concentration results were similar to the ash mass values. The BD concentrations did not significantly differ from the lowest DCP, NE, and N supplementation levels for the foot ash concentration and the lowest DCP supplementation level for the tibia ash concentration.

### Slope Ratios

The regression lines for N were nonlinear for traits pcdP and  $\text{InsP}_6$  disappearance, whereas the regressions for DCP and NE were linear for these traits. Therefore, linear regression lines were calculated for all supplements so that they could be compared. All the regression lines were linear for the bone ash traits (Table 4). An exponential regression was found to be the most suitable for ADG. The slope ratios (DCP/NE, DCP/N, and NE/N) differed among the traits (Figure 1). The activity of NE causing the equivalent response of 1 g P from DCP (DCP/NE) varied between 208 FTU (ADG) and 349 FTU (foot ash concentration), and the activity of N causing an equivalent response of 1 g P from DCP (DCP/N) varied from 357 FTU (ADG) to 640 FTU

**Table 2.** Initial BW (d 7), final BW (d 21/22), ADG, ADFI, and G:F of broiler chickens fed the experimental diets<sup>1</sup> (least square means and pooled SEM,  $n = 6$ ).

Trait	BD	DCP 0.7	DCP 1.4	DCP 2.1	DCP 2.9	NE250	NE500	NE750	N250	N500	N1000	Pooled SEM	P-value ANOVA
Initial BW (g)	181	183	178	180	179	183	182	181	185	184	181	5.4	0.998
Final BW (g)	545 <sup>e</sup>	640 <sup>d</sup>	755 <sup>c</sup>	812 <sup>b</sup>	876 <sup>a</sup>	677 <sup>d</sup>	806 <sup>b</sup>	821 <sup>b</sup>	659 <sup>d</sup>	737 <sup>c</sup>	824 <sup>b</sup>	20.4	<0.001
ADG (g)	22 <sup>f</sup>	31 <sup>e</sup>	40 <sup>c</sup>	44 <sup>b</sup>	48 <sup>a</sup>	34 <sup>d</sup>	43 <sup>b</sup>	44 <sup>b</sup>	32 <sup>de</sup>	38 <sup>c</sup>	44 <sup>b</sup>	0.9	<0.001
ADFI (g)	40 <sup>e</sup>	48 <sup>d</sup>	57 <sup>c</sup>	62 <sup>b</sup>	67 <sup>a</sup>	51 <sup>d</sup>	61 <sup>b</sup>	62 <sup>b</sup>	49 <sup>d</sup>	56 <sup>c</sup>	63 <sup>b</sup>	1.3	<0.001
G:F (g/g)	0.57 <sup>e</sup>	0.65 <sup>d</sup>	0.70 <sup>bc</sup>	0.71 <sup>ab</sup>	0.72 <sup>a</sup>	0.66 <sup>d</sup>	0.70 <sup>ab</sup>	0.71 <sup>ab</sup>	0.65 <sup>d</sup>	0.68 <sup>c</sup>	0.70 <sup>a</sup>	0.006	<0.001

<sup>1</sup>BD: basal diet, DCP: dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G.

<sup>a-f</sup>Means without a common superscript in each line are significantly different according to Fisher's LSD test ( $\alpha = 0.05$ ).

**Table 3.** Prececal phosphorus and calcium digestibility, prececal digestible P (pcdP), InsP<sub>6</sub> disappearance ( $n = 6$ ), and tibia and foot ash contents of broiler chickens fed the experimental diets<sup>1</sup> ( $n = 24$ ; least square means and pooled SEM).

Trait	BD	DCP 0.7	DCP 1.4	DCP 2.1	DCP 2.9	NE250	NE500	NE750	N250	N500	N1000	Pooled SEM	P-value ANOVA
InsP <sub>6</sub> disappearance (%)	60 <sup>e</sup>	47 <sup>dl</sup>	35 <sup>e</sup>	26 <sup>f</sup>	19 <sup>g</sup>	62 <sup>c</sup>	69 <sup>b</sup>	76 <sup>a</sup>	66 <sup>bc</sup>	60 <sup>c</sup>	76 <sup>a</sup>	2.3	<0.001
P digestibility (%)	58 <sup>cl</sup>	52 <sup>e</sup>	46 <sup>f</sup>	44 <sup>fg</sup>	41 <sup>g</sup>	57 <sup>dl</sup>	62 <sup>bc</sup>	66 <sup>a</sup>	59 <sup>cd</sup>	57 <sup>d</sup>	65 <sup>ab</sup>	1.3	<0.001
pcdP (g/kg DM)	2.59 <sup>cd</sup>	2.66 <sup>cd</sup>	2.67 <sup>cd</sup>	2.91 <sup>ab</sup>	3.03 <sup>a</sup>	2.55 <sup>d</sup>	2.76 <sup>bc</sup>	2.97 <sup>a</sup>	2.62 <sup>cd</sup>	2.53 <sup>d</sup>	2.92 <sup>ab</sup>	0.06	<0.001
pcdP intake (mg/day)	102 <sup>g</sup>	126 <sup>f</sup>	152 <sup>d</sup>	179 <sup>bc</sup>	203 <sup>a</sup>	129 <sup>ef</sup>	168 <sup>c</sup>	184 <sup>b</sup>	129 <sup>ef</sup>	142 <sup>de</sup>	183 <sup>b</sup>	4.6	<0.001
Ca digestibility (%)	68 <sup>ab</sup>	58 <sup>d</sup>	53 <sup>e</sup>	49 <sup>f</sup>	42 <sup>g</sup>	63 <sup>c</sup>	62 <sup>c</sup>	65 <sup>bc</sup>	70 <sup>a</sup>	63 <sup>c</sup>	65 <sup>bc</sup>	1.4	<0.001
Tibia ash (mg)	384 <sup>g</sup>	468 <sup>f</sup>	582 <sup>cd</sup>	672 <sup>b</sup>	783 <sup>a</sup>	495 <sup>ef</sup>	658 <sup>b</sup>	686 <sup>b</sup>	481 <sup>ef</sup>	528 <sup>de</sup>	634 <sup>bc</sup>	24.6	<0.001
Tibia ash (%)	34.2 <sup>e</sup>	35.6 <sup>de</sup>	39.7 <sup>c</sup>	42.0 <sup>b</sup>	44.4 <sup>a</sup>	36.7 <sup>d</sup>	40.8 <sup>bc</sup>	41.9 <sup>b</sup>	35.9 <sup>d</sup>	36.8 <sup>d</sup>	40.7 <sup>bc</sup>	0.74	<0.001
Foot ash (mg)	411 <sup>f</sup>	486 <sup>e</sup>	607 <sup>c</sup>	719 <sup>b</sup>	794 <sup>a</sup>	519 <sup>de</sup>	680 <sup>b</sup>	721 <sup>b</sup>	512 <sup>e</sup>	546 <sup>d</sup>	690 <sup>b</sup>	42.6	<0.001
Foot ash (%)	10.3 <sup>e</sup>	10.3 <sup>e</sup>	11.4 <sup>d</sup>	12.8 <sup>b</sup>	13.5 <sup>a</sup>	10.8 <sup>e</sup>	11.8 <sup>cd</sup>	12.5 <sup>b</sup>	10.5 <sup>e</sup>	10.6 <sup>e</sup>	12.4 <sup>bc</sup>	0.23	<0.001

<sup>1</sup>BD: basal diet, DCP: dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G.

<sup>a-g</sup>Means without a common superscript in each line are significantly different according to Fisher's LSD test ( $\alpha = 0.05$ ).

**Table 4.** Estimated parameters of the regressions calculated for the criteria of P evaluation<sup>1</sup>.

Trait	Type of Regression <sup>2</sup>	Common intercept	Slope DCP ( $\beta_{DCP}$ )	Slope NE ( $\beta_{NE}$ )	Slope N ( $\beta_N$ )	R <sup>2</sup>	RMSE
pcdP (mg/kg DM)	linear	2485 (40.9)	187 (25.5)	0.56 (0.098)	0.32 (0.070)	0.49	162
pcdP (mg/animal/d)	linear	103 (2.73)	34.7 (1.62)	0.11 (0.006)	0.069 (0.004)	0.90	10.2
InsP <sub>6</sub> disappearance (%)	linear	56.9 (1.51)	-13.7 (0.94)	0.024 (0.0036)	0.015 (0.0026)	0.91	6.00
Tibia ash (mg)	linear	393 (18.1)	133 (7.49)	0.42 (0.031)	0.22 (0.022)	0.58	99.1
Tibia ash (% DM)	linear	33.9 (0.37)	3.71 (0.232)	0.011 (0.0009)	0.0058 (0.0006)	0.52	2.99
Foot ash (mg)	linear	415 (16.5)	135 (7.0)	0.43 (0.030)	0.24 (0.022)	0.60	95.8
Foot ash (% DM)	linear	9.79 (0.139)	1.29 (0.084)	0.0037 (0.0003)	0.0021 (0.0002)	0.53	1.03
ADG (g/d)	exponential	22.0 (1.35)	0.50 (0.171)	0.0024 (0.00096)	0.0014 (0.00055)	0.80	3.41

<sup>1</sup>BD: basal diet, DCP: dicalcium phosphate, NE: Natuphos E 5000 G, N: Natuphos 5000 G, pcdP: prececal digestible P. Standard errors of the estimates are shown in parentheses.

<sup>2</sup>Linear regression equation:  $y_{ij} = \mu + \beta_{NE}C_{NEij} + \beta_N C_{Nij} + \beta_{DCP}C_{DCPij} + b_j + e_{ij}$ ; Exponential regression equation:  $y_{ij} = a_s + (\mu - a_s) \cdot [1 - e^{-\beta_{NE} \cdot C_{NEij}} - e^{-\beta_N \cdot C_{Nij}} - e^{-\beta_{DCP} \cdot C_{DCPij}}] + b_j + e_{ij}$ . Plateau DCP = 55.6 (5.98), Plateau NE = 49.5 (5.43), Plateau N = 49.0 (4.99).

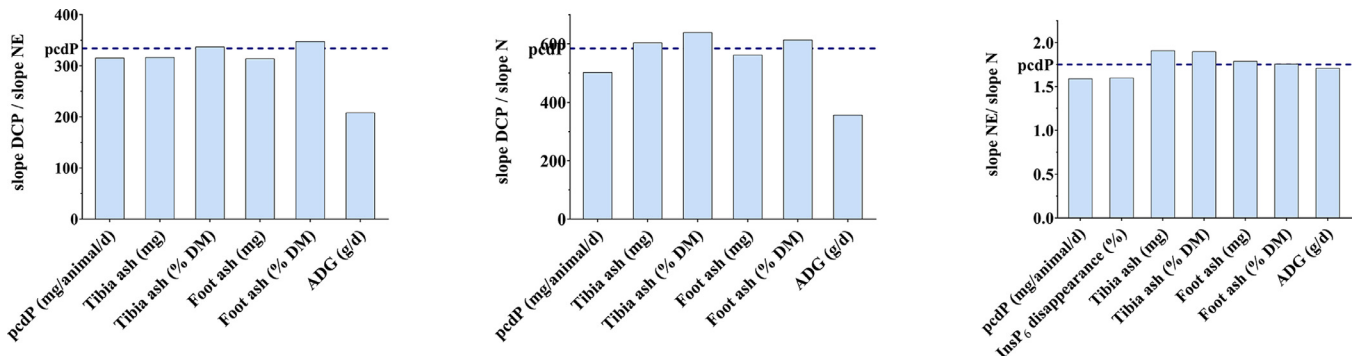
(tibia ash concentration). The ratio of responses between the 2 phytase products (NE/N) varied from 1.59 (pcdP intake) to 1.91 (tibia ash mass).

## Microbiota Responses

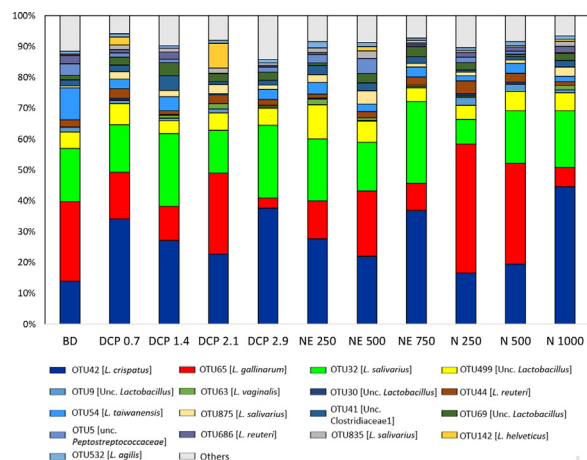
An overall significant effect of treatment on ileal microbial composition was detected by the PERMANOVA analysis ( $P = 0.023$ ). Pairwise comparisons including Benjamini Hochberg correction did not show any significant difference among the diets (Table S2). The alpha diversity calculations did not result in a significant difference when the Shannon diversity, Simpson, and Pielou's evenness indices were used (Figure S1). The average similarity among all experimental treatments ranged from 35-69% (Table S3).

The dominant genus in this study was *Lactobacillus*, with a relative abundance of between 97% in N 1000 and 84% in NE 500. The relative abundance distribution showed that OTU42 (*Lactobacillus crispatus*) and OTU32 (*Lactobacillus salivarius*) increased as the supplementation levels rose (Figure 2). In contrast, OTU65 (*Lactobacillus gallinarum*) decreased at the highest supplementation levels compared to the lower levels.

*Lactobacillus crispatus* (OTU42) was the most abundant single OTU and the highest value (44.5%) was observed when N supplementation was greatest (Figure 2). The lowest OTU42 abundance was found in BD (14%). This OTU was positively correlated with all performance traits, pcdP, and all ash traits ( $R = 0.245 - 0.321$ ,  $P = 0.010 - 0.050$ ; Figure S2, Table S4). The OTU with the second-highest abundance was assigned to *L. gallinarum* (OTU65) and its abundance was



**Figure 1.** Equivalence calculations for the 3 supplements and the various response traits. Dotted lines show the equivalence calculated based on the concentration of prececal digestible P (pcdP) in the diet. Abbreviations: DCP, Dicalcium phosphate; NE, Natuphos E 5000 G; N, Natuphos 5000 G.



**Figure 2.** Relative abundance (%) of the most prevalent OTUs in the ileal content of the birds fed the experimental treatments. Abbreviations: BD, Basal diet; DCP, Dicalcium phosphate; NE, Natuphos E 5000 G; N, Natuphos 5000 G.

greatest (42%) when the N supplementation level was lowest. Operational taxonomic unit 65 had negative correlations with final BW, G:F, and the ash traits ( $R = -0.236 - 0.298$ ,  $P = 0.016 - 0.050$ ) (Table S4). *Lactobacillus salivarius* (OTU32) was the OTU with the third-highest abundance and was mainly present in treatment NE 750 (27%). Due to missing information in public databases, many of the sequences could not be assigned to a specific species. For example, an unclassified *Lactobacillus* (OTU499) had an abundance of 4% – 11% in treatments DCP 1.4 and NE 250, respectively. Another unclassified *Lactobacillus* (OTU9) had a very low abundance in all NE treatments (0.14 – 0.4%) and DCP 2.9 (0.4%), but its abundance increased in N 250 and N 500 (both 2.5%). Other detected species, with a maximum abundance of 4.7%, belonged to unclassified Clostridiaceae 1, *Streptococcus*, and *Enterococcus azikevi*.

## DISCUSSION

### Evaluation Traits

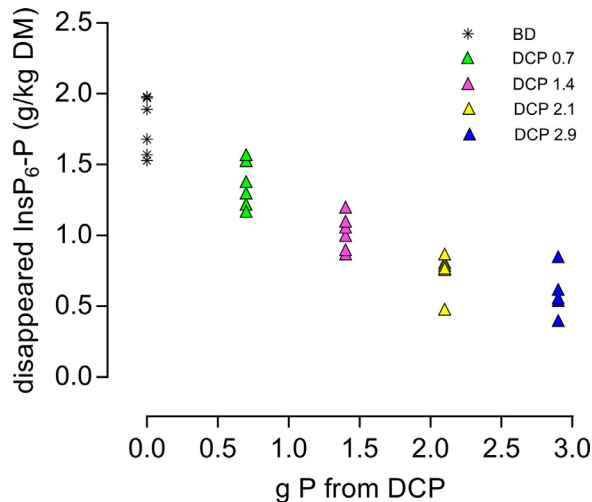
One hypothesis of this study was that different response traits cause different estimates of relative efficacy. The results confirmed this hypothesis. Based on the suggestions made by WPSA (2013), pcdP was considered the reference trait and other traits were set in relation to pcdP. The traits providing the most consistent slope ratios with the smallest deviation from pcdP in this study were foot ash concentration and foot ash mass (Figure 1). Shastak et al. (2012a) also used foot ash mass when they undertook a regression analysis to compare different mineral P sources, but they used dissected bones without adhering tissues. Shastak et al. (2012a) found that foot ash mass was the trait with the second lowest slope ratio deviations compared to P retention after pcdP when the birds were 35 d old. In the present study, the foot contained more ash mass than the tibia in all treatments, but the variation in values was lower. Therefore, analysis of the foot may

provide more accurate results than the tibia when used for phytase and P evaluation purposes. Using the foot inclusive of adhering tissues has an additional advantage over the tibia because it is less laborious to analyze.

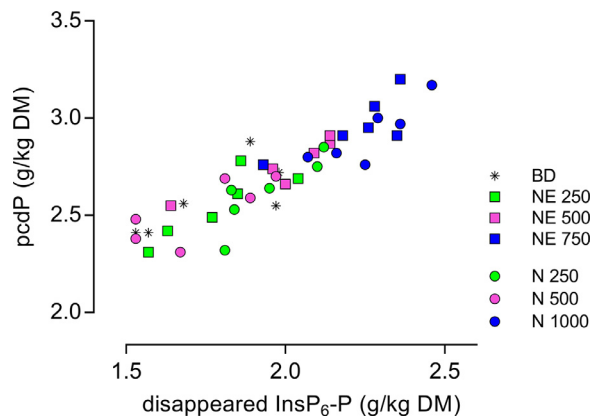
In addition to bone data, ADG has been used as an easy-to-determine response trait in relative bioavailability studies. Denbow et al. (1995) reported that BW gain is an even more sensitive trait than tibia ash concentration for determining relative P availability. In the present study, the response ratios were very similar for ADG and pcdP when NE and N were compared, but not when the enzymes were compared to DCP (Figure 1). Létourneau-Montminy et al. (2010) reported that most of the P in an animal is retained in the bones and the amount of digestible P needed to achieve high growth performance is lower than that needed to achieve high tibia ash concentrations. This finding was confirmed by the results from the present study. An exponential function yielded the best fit for the ADG responses, whereas linear functions best fitted the pcdP and ash traits results (Table 4), which indicated that bone mineralization in the birds responded to incremental supply, although there was no further gain in BW.

The pcdP value is often expressed as a concentration (% or g/kg feed), but pcdP intake (mg/d) can also be calculated. The pcdP intake may reflect the amount of P actually retained by the animal, assuming that any excretion of P via urine is very low at the P supply level used in this study (Rodehutsord et al., 2012). However, the deviations of slope ratios of pcdP intake from pcdP concentration were ranked at positions 2–6 for the analyzed traits, which may be because the feed intake was being affected by more factors than P digestibility. This suggests that pcdP expressed as a concentration appears to be a more appropriate measure for P evaluation than pcdP intake. The mean of all response traits shown in Figure 1 leads to the conclusion that a 1.75-fold increase in the activity of phytase N is needed to achieve the same response as NE.

For  $\text{InsP}_6$  disappearance, only the ratio between the 2 phytase products was calculated because supplemented DCP negatively affected prececal  $\text{InsP}_6$  disappearance in a nonlinear fashion (Figure 3). Deviation from linearity only occurred at the highest DCP inclusion level. With the exception of this one treatment, the relationship between  $\text{InsP}_6$ -P disappearance ( $y$ , g/kg DM) and P from DCP ( $x$ , g/kg DM) was described by the following linear regression:  $y = 1.72$  ( $SE$  0.05) – 0.48 ( $SE$  0.04) $x$ ;  $RMSE = 0.16$ ,  $R^2 = 0.86$ ), which indicates that  $\text{InsP}_6$ -P digestibility decreased by 0.48 g for each 1 g of supplemented DCP. This estimate is consistent with estimates from other studies where  $\text{InsP}_6$ -P digestibility was reduced by 0.4 to 0.5 g/kg DM for each gram of mineral P supplementation per kg of diet (Rodehutsord, 2016) and confirms the detrimental effect of using mineral P sources for phytate utilization by broiler chickens. A consistent linear relationship between  $\text{InsP}_6$ -P disappearance and P digestibility was found when the BD and the phytase-containing diets were analyzed together (Figure 4). The estimated slope of the



**Figure 3.** Relationship between the amount of supplemented P from dicalcium phosphate (DCP) and  $\text{InsP}_6\text{-P}$  disappearance until the end of the ileum for the basal diet (BD) and the different DCP supplementation levels. The relationship is described by the exponential function  $y = 1.77$  ( $SE = 0.06$ ) +  $(0.06$  ( $SE = 0.35$ ) -  $1.77$  ( $SE = 0.06$ ))  $\times$  ( $1 - \exp(-0.43$  ( $SE = 0.15$ )  $x$ );  $RMSE = 0.15$ ,  $R^2 = 0.90$ .



**Figure 4.** Relationship between  $\text{InsP}_6\text{-P}$  disappearance and P digestibility until the end of the ileum for the basal diet (BD), and the different supplementation levels of Natuphos E 5000 G (NE) and Natuphos 5000 G (N). Each data point represents a pen. Linear regressions were calculated together for all treatments as there were no significant differences between the single regressions ( $P \geq 0.563$ ). The relationship is described by the linear function  $y = 1.15$  ( $SE = 0.16$ ) +  $0.79$  ( $SE = 0.08$ )  $x$ ;  $RMSE = 0.10$ ,  $R^2 = 0.81$ .

relationship indicated that 0.79 g of digestible P was provided by 1 g of  $\text{InsP}_6\text{-P}$  that was additionally degraded when the animal was given a phytase supplement, irrespective of the phytase product. This estimate is almost the same as the 0.78 calculated from previous studies when phytase supplements were added to several corn- and wheat-based diets (Rodehutschord, 2016). Overall, this indicates that out of all the  $\text{InsP}_6$  that is initially hydrolyzed, about 20% of the P remains indigestible, probably because it is bound to lower inositol phosphates. In the present study and in previous studies from our laboratory (Zeller et al., 2015; Sommerfeld et al., 2018), ileal concentrations of some  $\text{InsP}_4$  and  $\text{InsP}_3$  isomers were higher in phytase-supplemented diets than in BD (Table S1). As expected, ileal

concentrations of  $\text{Ins}(1,2,4,5,6)\text{P}_5$  were higher after supplementation with N compared to NE, while the opposite was found for  $\text{Ins}(1,2,3,4,5)\text{P}_5$ , which reflected their classification as 3-phytase and 6-phytase, respectively. Differences between the 2 phytases were also observed in the ileal concentrations of  $\text{Ins}(1,2,3,4)\text{P}_4$ . However, there was no difference between the 2 phytase products with regard to the total amount of P that remained bound to lower inositol phosphates. This indicates that the differences in efficacy of the 2 phytases (Figure 1) are only related to the initial dephosphorylation of the  $\text{InsP}_6$  molecule.

### Ileal Microbiota Composition

The second hypothesis was that ileal microbiota composition is differentially affected by phytases and DCP. This hypothesis was not confirmed by the present data. Effects of phytase supplementation on microbiota composition due to P and Ca release from phytate have been previously reported (Witzig et al., 2015; Borda-Molina et al., 2016). In the present study, higher levels of phytases led to similar microbial ecology distributions as high concentrations of P from DCP. Bacterial species that are highly dominant in a defined environment, such as the ileum, may show reduced interactions with other microorganisms and this could improve nutrient assimilation efficiency (Larsen and Claassen, 2018).

*Lactobacillus* was found to be highly dominant in the present study, which was consistent with previous results (Witzig et al., 2015; Borda-Molina et al., 2016). The high dominance might be a reason for the low  $\alpha$ -diversity. However, Witzig et al. (2015) and Borda-Molina et al. (2016) reported a higher diversity among treatments than in the present study. This may have been related to the use of other phytase products or different supplementation levels compared to the present study. Species such as *L. reuteri*, *L. amylovorus*, *L. salivarius*, and *L. crispatus*, which are known to have phytase-like activity (Raghavendra and Halami, 2009; Lee et al., 2013; Nuobariene et al., 2015; NCBI, 2016), were found in all treatments, but their proportions were not equal. However, there was no apparent connection to the supplement and supplementation levels. The abundance of *L. crispatus* was lowest in BD where a high microbial phytase-like activity was expected. Furthermore, *L. crispatus* abundance was highest in N 1000 where a decrease in microbial phytase-like activity due to high phytase supplementation was expected. Therefore, microbial phytase-like activity seemed to have a lower effect on  $\text{InsP}_6$  degradation than mucosal phytase in the present study. Previous studies have suggested that among the endogenous enzymes, mucosa-derived phytases are more important than phytases produced by bacteria (Künzel et al., 2019; Sommerfeld et al., 2019).

The 2 most abundant OTUs, *L. crispatus* and *L. gallinarum*, showed significant correlations with performance and ash traits. *Lactobacillus crispatus* was positively correlated with these traits whereas *L.*



*gallinarum* showed negative correlations. These results were stimulated by the high inclusion levels of the respective supplements. Both strains have been reported to have functional adherence capabilities, are highly effective at colonizing the ileum, and show bile tolerance (Spivey et al., 2014). These could be compensatory effects in ecological terms and would increase the presence of the strain, possibly because of effective repeated colonization of the chicken gut due to positive responses to host genetics and environmental factors, including diet (Stephenson et al., 2010).

The conclusions of this study are that different response traits cause different estimates of relative phytase efficacy. The average values for the response traits showed that a 1.75-fold increase in the activity of phytase N is needed to achieve the same response as NE. The supplementation of DCP or either of the phytase products did not have a major effect on the microbiota composition. Similar composition was driven by the high relative abundances of *L. crispatus*, *L. salivarius*, and *L. gallinarum*.

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## DISCLOSURES

D. F. is an employee of BASF SE. The authors declare that they have no competing interests.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.psj.2021.101133](https://doi.org/10.1016/j.psj.2021.101133).

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