Animal Microbiome

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Effects of calcium level and source, formic acid, and phytase on phytate degradation and the microbiota in the digestive tract of broiler chickens

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Abstract

Background: Diet acidification, dietary calcium (**Ca**) level, and phytase supplementation are known influences on the microbial community in the digestive tract and on phosphorus (**P**) utilization of broiler chickens. Effects of dietary factors and microbiota on P utilization may be linked because microorganisms produce enzymes that release P from phytate (**$InsP_6$**), the main source of P in plant feedstuffs. This study aimed to detect linkages between microbiota and $InsP_6$ degradation by acidifying diets (i.e., replacing Ca carbonate (**$CaCO_3$**) by Ca formate or adding formic acid to $CaCO_3$-containing diets), varying Ca levels, and supplementing phytase in a three-factorial design. We investigated i) the microbial community and pH in the digestive tract, ii) prececal (**pc**) P and Ca digestibility, and iii) $InsP_6$ degradation.

Results: All factors under investigation influenced digesta pH and the microbiota composition. Predicted functionality and relative abundance of microorganisms indicated that diets influenced the potential contribution of the microbiota on InsP degradation. Values of $InsP_6$ degradation and relative abundance of the strains *Lactobacillus johnsonii* and *Lactobacillus reuteri* were correlated. Phytase supplementation increased pc $InsP_6$ disappearance, with differences between Ca levels, and influenced concentrations of lower inositol phosphate isomers in the digestive tract. Formic acid supplementation increased pc $InsP_6$ degradation to *myo*-inositol. Replacing $CaCO_3$ by Ca-formate and the high level of these Ca sources reduced pc $InsP_6$ disappearance, except when the combination of $CaCO_3$ + formic acid was used. Supplementing phytase to $CaCO_3$ + formic acid led to the highest $InsP_6$ disappearance (52%) in the crop and increased *myo*-inositol concentration in the ileum digesta. Supplementing phytase leveled the effect of high Ca content on pc $InsP_6$ disappearance.

Conclusions: The results point towards a contribution of changing microbial community on $InsP_6$ degradation in the crop and up to the terminal ileum. This is indicated by relationships between $InsP_6$ degradation and relative abundance of phosphatase-producing strains. Functional predictions supported influences of microbiota on $InsP_6$ degradation. The extent of such effects remains to be clarified. $InsP_6$ degradation may also be influenced by variation of pH caused by dietary concentration and solubility of the Ca in the feed.

Keywords: Calcium, Phytate, Microbiota, Functionality, Broiler chickens

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Background

High utilization of phosphorus (**P**) provided by plant feedstuffs is advantageous because less or no mineral P is needed to fulfill the P requirement of animals. Plant-P is mainly bound in phytic acid [*myo*-inositol 1,2,3,4,5, 6-hexakis (dihydrogen phosphate); **$InsP_6$**] and present as phytate, which requires hydrolyzing enzymes to make P available for the animal. Hence, phytases are widely used as feed additives in non-ruminant nutrition. The main effect of phytases is the cleavage of P from $InsP_6$ to increase P utilization by animals. When diets are fed with the supplementation of mineral P and calcium (**Ca**), but without supplemented phytase, prececal (**pc**) $InsP_6$ degradation is reduced in broiler chickens [1]. The reduced degradation of $InsP_6$ upon Ca supplementation is usually explained, among other reasons, by the formation of Ca-$InsP_6$ complexes in the digestive tract at high Ca concentrations [2, 3].

It was shown that bone mineralization of broiler chickens can differ as a result of varying Ca sources when supplied in equal Ca concentrations [4] and that using different Ca sources can have other consequences, such as impacts on the microbial community of the digestive tract of broiler chickens [5]. Studies investigating the impact of dietary Ca on P utilization usually used limestone as a Ca source, which mainly consists of calcium carbonate (**$CaCO_3$**), a compound with a high buffer and acid-binding capacity. Increasing pH in the digestive tract supports formation of Ca-$InsP_6$ complexes and, thus, reduces pc $InsP_6$ degradation [2, 6, 7]. Reducing intestinal pH might counteract effects of high dietary Ca concentrations. Ca salts of organic acids like Ca-formate are known to have a lower buffer capacity than $CaCO_3$ [8]. Hence, replacing $CaCO_3$ by Ca-formate might compensate the pH increase caused by $CaCO_3$ supplementation and thereby increase $InsP_6$ degradation and, as a consequence of changing the Ca source, affect the microbial community. Solubility of dietary Ca might also influence P utilization through Ca-$InsP_6$ complexes or other mechanisms. Complexation of Ca and $InsP_6$ was shown to be increased at pH 5 and higher [9]. Using Ca sources with higher solubility than that of $CaCO_3$ might allow for a higher Ca absorption in the proximal section of the small intestine and thus reduce the amount of Ca cations available for Ca-$InsP_6$ complex formation.

A decrease in pH could also be achieved by adding formic acid to diets. As reviewed by Kim et al. [10], supplementation of organic acids was reported to decrease pH particularly in the crop, with some studies describing decreased pH up to the ileum. The organic acid formic acid, was reported to affect the microbial community and lead to increased performance and nutrient digestibility in broiler chickens [10]. Likely, the addition of formic acid to $CaCO_3$-containing diets and the use of Ca-formate influences the microbial community in the digestive tract, possibly through changes in digesta pH [11, 12]. Therefore, it is possible that different microbiota composition is involved in causing the observed effects of Ca sources, organic acid supplementation, and Ca levels on P utilization.

The present study was conducted to investigate effects of diet acidification, dietary Ca, and phytase supplementation on the microbiota of the crop and the terminal small intestine of broiler chickens based on target amplicon sequencing of DNA, and to study consequences on pc $InsP_6$ disappearance, and pc P and Ca digestibility. We used a $3 \times 2 \times 2$ factorial design for the experiment (Table 1) with two Ca levels (5.6 and 8.2 g/kg dry matter) and two phytase supplementation levels (0 and 1500 FTU/kg). The third factor was the addition of 6 g formic acid/kg to $CaCO_3$-containing diets or replacing $CaCO_3$ by Ca-formate, which is termed "acidification" herein. By now, most studies only investigated one or two of these factors, making potential interactions between the three factors impossible to detect. This makes the present study the first to examine the influence of phytase and acidifying ingredients with different Ca levels on P utilization following $InsP_6$ degradation, the microbial community, and potential linkages between all traits. To elucidate the underlying mechanisms, pH and inositol phosphate (**InsP**) isomers in the digesta of the crop, gizzard, and terminal small intestine were analyzed. It was hypothesized that replacing $CaCO_3$ by Ca-formate or adding formic acid to $CaCO_3$-containing diets decreases the pH, influences the microbial community, and increases $InsP_6$ degradation and P digestibility.

Table 1 Description of 12 dietary treatments in a $3 \times 2 \times 2$ experimental design

Acidification	Ca level (g/kg dry matter)	Phytase supplementation (FTU/kg)
$CaCO_3$	5.6	0
		1500
	8.2	0
		1500
$CaCO_3$ + formic acid	5.6	0
		1500
	8.2	0
		1500
Ca-formate	5.6	0
		1500
	8.2	0
		1500

Results

The initial group weight at the beginning of feeding the experimental diets, on day 16 of life, was similar for all treatments, at 9604 g/pen (standard deviation = 15 g/pen, $P = 0.183$). The experimental period ended on day 21 and day 22 post-hatch for half of the replicate pens of each treatment. Mortality was low (0.6% of all animals) and not related to any treatment (six cases in six different treatments).

Growth performance

The average daily gain (**ADG**) and average daily feed intake (**ADFI**) during the experimental period were lower compared to other treatments when the Ca level was high and no phytase was supplemented ($P < 0.001$; Fig. 1, Table S1). The ADFI was significantly lower for Ca-formate compared to $CaCO_3$ and $CaCO_3$ + formic acid ($P = 0.002$). Phytase supplementation increased the gain to feed ratio (**G:F**) by 0.04 g/g ($P < 0.001$). Further effects on growth performance were not significant.

pH in the digestive tract

The contents of crop, gizzard, and posterior small intestine were obtained immediately after slaughter of the birds. In the crop content, the highest pH of 5.5 was observed for $CaCO_3$. Crop pH was decreased by 0.6 and 0.3 units for $CaCO_3$ + formic acid and Ca-formate, respectively, compared to $CaCO_3$ ($P < 0.001$; Fig. 2, Table S2). With a 0.1-unit increase, crop pH was marginally but significantly higher for the high compared to the low Ca level. In the gizzard, phytase supplementation increased pH by 0.2 units ($P < 0.001$) and decreased pH by 0.1 units ($P = 0.005$) at the low and high Ca level, respectively. In the ileum, phytase supplementation increased pH by 0.8 units at the high Ca level ($P < 0.001$) but had no significant effect at the low Ca level.

Microbial community

In content of both the crop and the ileum, mainly *Lactobacillus* species, including *L. johnsonii*, *L. crispatus, L. reuteri, L. gallinarum,* and *L. vaginalis*, were identified (Fig. 3 and Fig. 4). *Streptococcus alactolyticus* was highly abundant in the crop and the ileum for diets with $CaCO_3$ at the low Ca level, irrespective of phytase supplementation. This was reflected by a high similarity between $CaCO_3$ treatments at the low Ca level and a separation of these treatments from the other diets in a cluster analysis (Fig. S1). Permutational multivariate analysis of variance (**PERMANOVA**) analyses (Table S3) revealed that the microbial communities in the crop and the ileum were significantly affected by all the main effects ($P \leq 0.034$), with no interaction being significant. The microbial community in birds receiving $CaCO_3$ differed significantly in both sections of the digestive tract compared to $CaCO_3$ + formic acid or Ca-formate ($P \leq 0.005$).

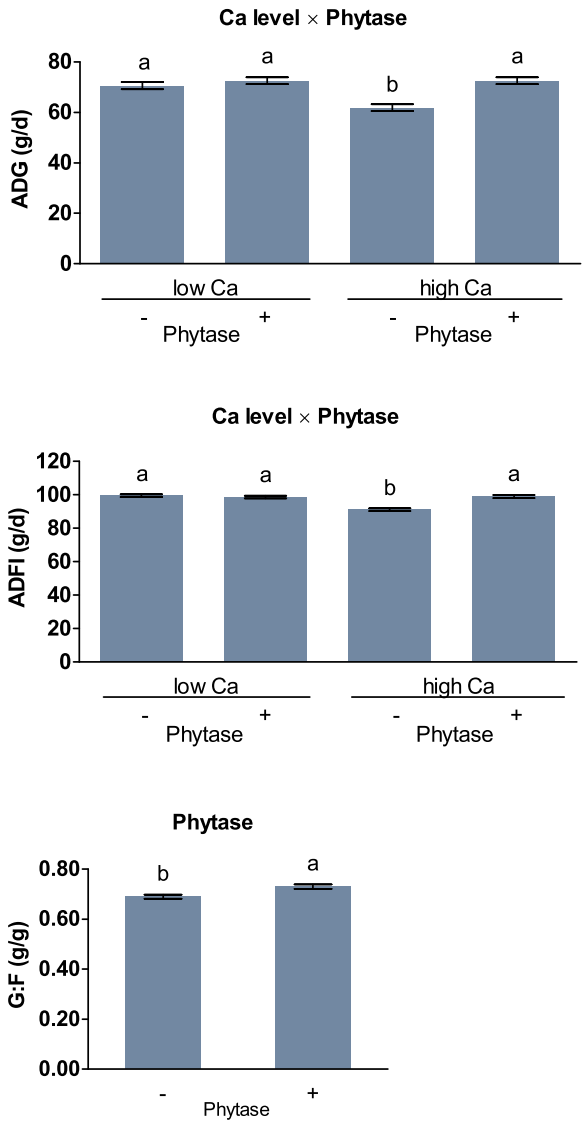

Fig. 1 Average daily gain (ADG), average daily feed intake (ADFI), and the gain to feed ratio (G:F) of broiler chickens fed with differently acidified diets with low and high Ca levels and without (–) or with (+) supplementation of 1500 FTU phytase/kg fed from day 16 of life to the end of the experiment on day 21 or 22 for half of the replicate pens of each treatment. Only significant ($P < 0.050$) effects are shown. Columns within a statistical comparison not sharing the same letter are significantly different ($P \leq 0.050$). Details of the statistical evaluations are shown in Table S1

In the crop content, between 34 and 53% of the detected relative abundance of microorganisms corresponded to operative taxonomic unit (**OTU**) 1 (*L. johnsonii*) and between 11 and 29% to OTU2 (*L. gallinarum*). We conducted analyses of variance (**ANOVA**) to get information on treatment effects on single OTUs being aware that explanatory power is

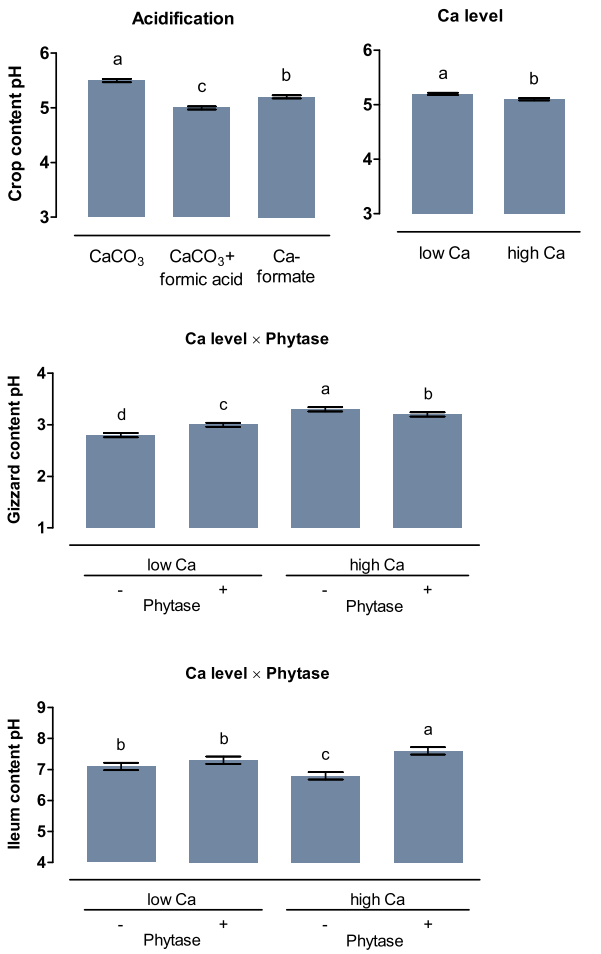

Fig. 2 pH in the crop, gizzard, and ileum of broiler chickens fed with differently acidified diets with low and high Ca levels and without (–) or with (+) supplementation of 1500 FTU phytase/kg. Only significant ($P < 0.050$) effects are shown. Columns within a statistical comparison not sharing the same letter are significantly different ($P \leq 0.050$). Details of the statistical evaluations are shown in Table S2

partly impinged by physiological interrelationships between microorganisms. Abundance of OTU1 (*L. johnsonii*) was higher for Ca-formate when phytase was supplemented ($P \leq 0.050$) (Table S4). The abundance of OTU1 increased for Ca-formate at the high Ca level. $CaCO_3$ increased the abundance of the OTUs assigned to *L. reuteri* (OTU9, OTU11, OTU15, and OTU19, $P \leq 0.001$). The high Ca level led to an increased abundance of OTU4 ($P = 0.005$), OTU9 ($P = 0.036$), and OTU11 ($P < 0.001$), which were assigned to *L. reuteri*. Phytase supplementation increased the abundance of OTU9, OTU11, and OTU13 (*L. reuteri*). Further significant influences on OTUs assigned to *L. vaginalis*, *L. gallinarum*, *S. alactolyticus*, *Gallibacterium sp.*, Unc. Bacterium, and Unc. Firmicutes are shown in Table S4 and Table S5. Crop pH was positively correlated with seven out of nine OTUs assigned to *L. reuteri* ($P \leq 0.027$, Table S6, Fig. S2). Similar to the crop, the most abundant OTUs in the ileum were OTU1 (*L. johnsonii*, 24–46%) and OTU2 (*L. gallinarum*, 15–41%). Phytase supplementation increased the abundance of OTU1 ($P = 0.040$) and OTU2 ($P = 0.015$). The abundance of OTU2 was increased in the ileum of chicken fed with the high Ca level diets ($P = 0.009$). The abundance of OTUs assigned to *L. reuteri* (OTU6, OTU15, and OTU18, $P \leq 0.049$) were decreased for Ca-formate and the abundance of OTU19 (*L. reuteri*, $P = 0.049$) was increased for $CaCO_3$.

Six significant correlations ($P \leq 0.038$) with OTUs assigned to *L. johnsonii*, *L. gallinarum*, and *L. reuteri* were determined for $InsP_6$ concentration in the ileum, pc P digestibility, and pc $InsP_6$ disappearance (OTU1, OTU2, OTU4, OTU9, OTU11, and OTU13) (Table S7, Fig. S3). Correlations with OTUs assigned to Unc. *Firmicutes*, *L. reuteri*, *S. alactolyticus*, and *Gallibacterium* sp. were significant in five cases for pH in the ileum (OTU7, OTU9, OTU10, OTU11, and OTU23). Correlations with concentrations of *myo*-inositol in the ileum were significant for OTUs assigned to *L. johnsonii*, *L. gallinarum*, and *L. reuteri* (OTU1, OTU2, OTU9, OTU11, and OTU13).

Functional prediction

The broad classification hierarchy of KEGG pathways of functions showed that same P-related pathways were significantly influenced in the crop and the ileum (Fig. 5, Table S8). No interaction related to genes connected to InsP metabolism was significant in the crop and the ileum. In the crop, genes encoding for InsP metabolism were higher in the $CaCO_3$ than in the $CaCO_3$ + formic acid and Ca-formate treatments ($P \leq 0.001$) and more abundant at the low than at the high Ca level ($P = 0.007$). In the ileum, the abundance of genes related to InsP metabolism was lower in the Ca-formate treatment than in the $CaCO_3$ and $CaCO_3$ + formic acid treatments ($P \leq 0.011$) and not influenced by Ca level. Phytase supplementation had no effect on InsP metabolism pathways in the crop and the ileum. Other significantly influenced pathways were mineral absorption, the phosphotransferase system, the phosphatidylinositol signaling system, and phosphonate and phosphinate metabolism in the crop and the ileum. Phytase supplementation decreased the abundances of genes related to the phosphonate and phosphinate metabolism ($P = 0.048$) in the crop and increased the abundances of genes related to the phosphatidylinositol signaling system in the ileum ($P = 0.031$). The other pathways were influenced by acidification, Ca level, or the two-way interaction between acidification and Ca level with no apparent pattern in changes.

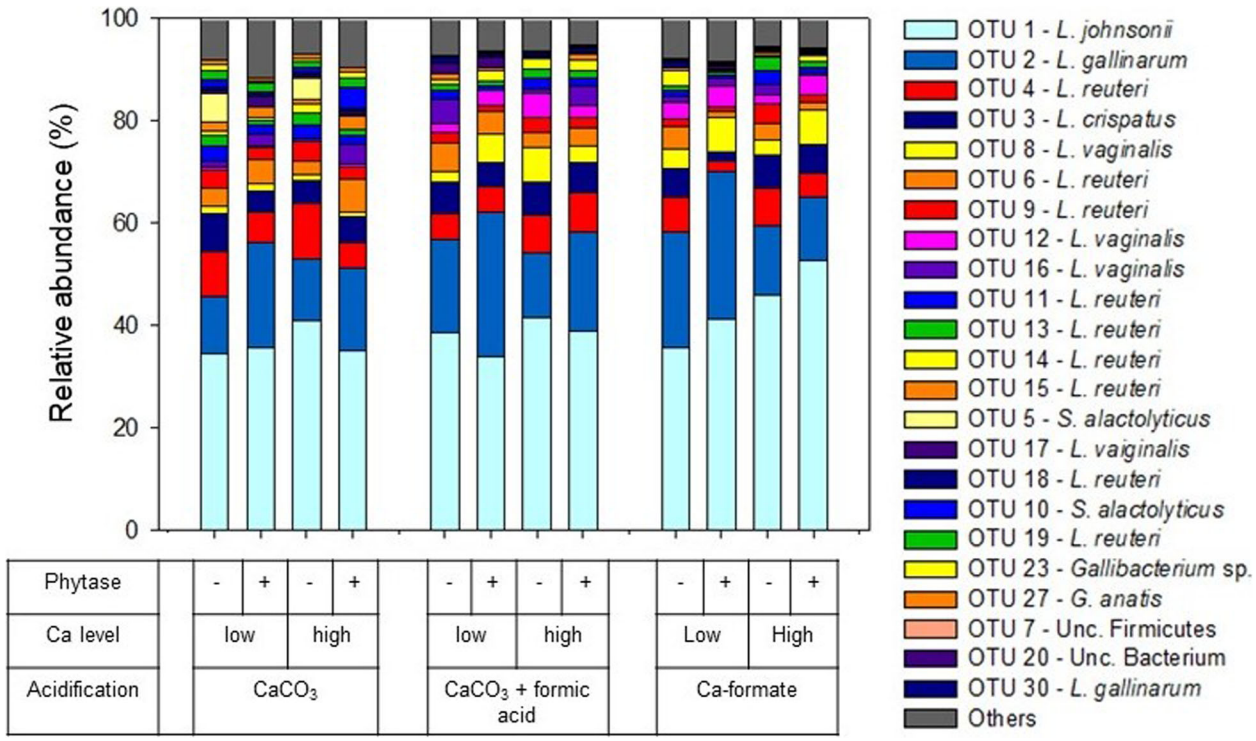

Fig. 3 Relative abundance of OTUs in the crop content of broiler chickens in the respective dietary treatments (only OTUs with a relative abundance > 1% are presented)

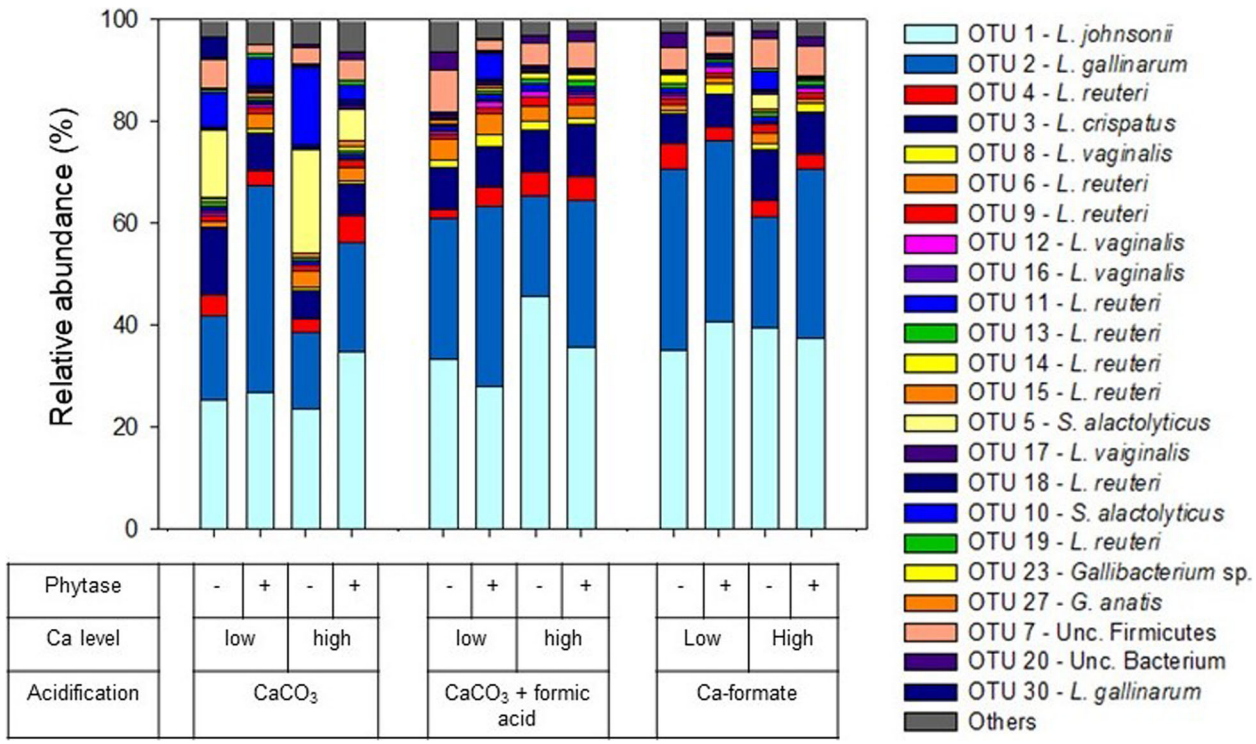

Fig. 4 Relative abundance of OTUs in the ileum digesta of broiler chickens in the respective dietary treatments (only OTUs with a relative abundance > 1% are presented)

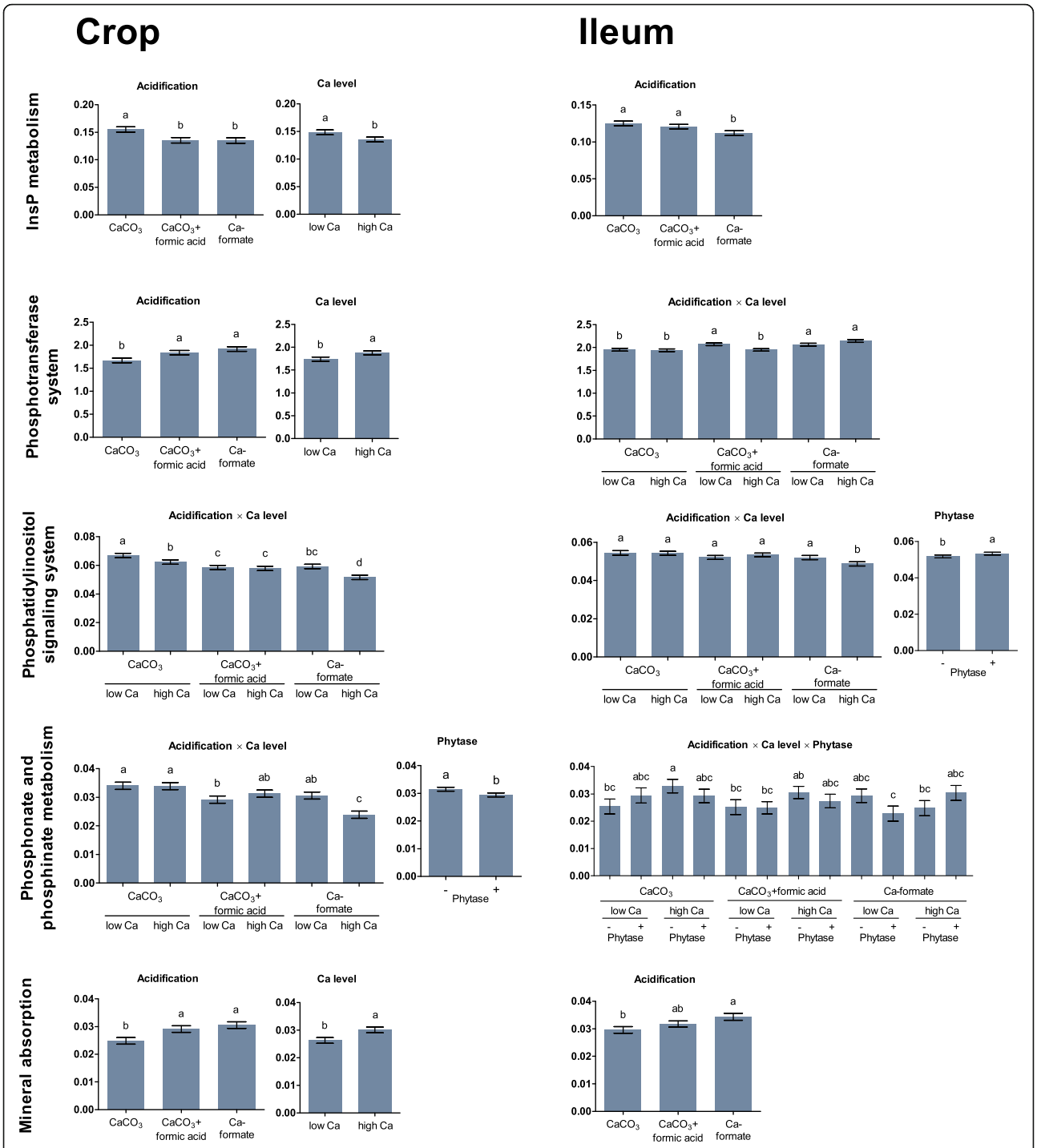

Fig. 5 Relative proportion of genes assigned to P-related KEGG pathways in the crop content and the ileum digesta of broiler chickens fed with differently acidified diets with low and high Ca levels without (–) and with (+) supplementation of 1500 FTU phytase/kg. Only significantly influenced pathways are presented ($P < 0.050$). Columns within a statistical comparison not sharing the same letter are significantly different ($P \leq 0.050$). Details of the statistical evaluations are shown in Table S8

Within the category of InsP metabolism, increasing the Ca level reduced abundance of genes coding for *myo*-inositol-1(or 4)-monophosphatase in the $CaCO_3$ and Ca-formate treatments ($P \leq 0.018$), but not in the $CaCO_3$ + formic acid treatment ($P = 0.520$) in the crop (Fig. 6, Table S9). In the ileum, *myo*-inositol-1(or 4)-monophosphatase was increased by phytase supplementation ($P = 0.040$). Increasing dietary Ca had no effect on

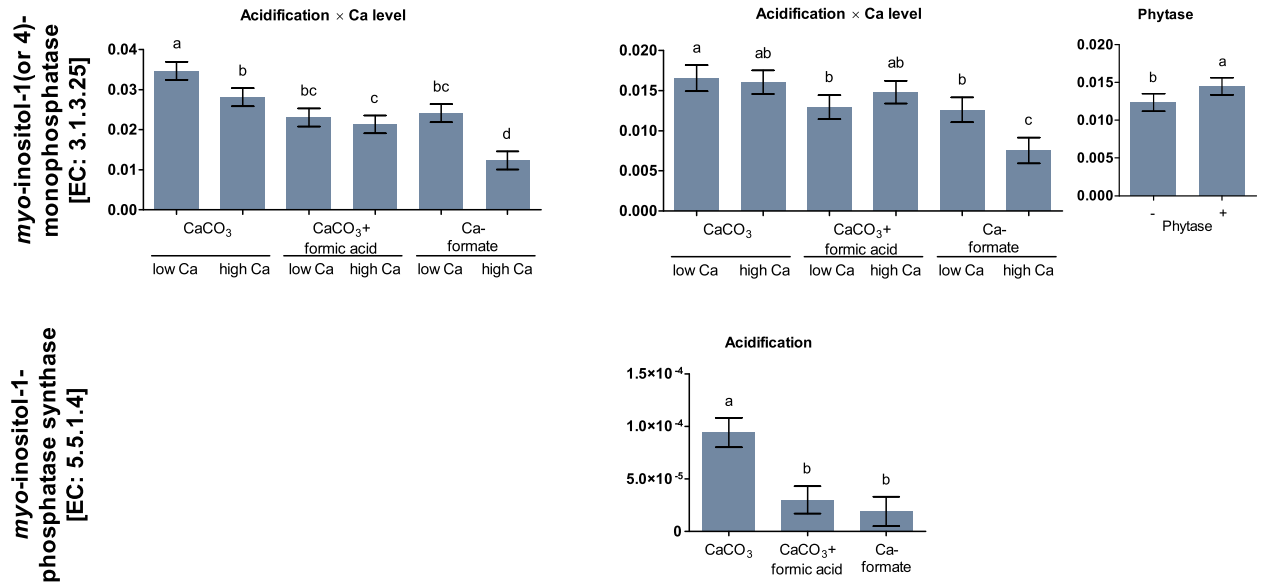

Fig. 6 Relative proportion of genes assigned to enzymes related to inositol phosphate and myo-inositol degradation listed in the KEGG database in the crop content and the ileum digesta of broiler chickens fed with differently acidified diets with low and high Ca levels without (–) and with (+) supplementation of 1500 FTU phytase/kg. Only significantly influenced enzymes are presented ($P < 0.050$). Columns within a statistical comparison not sharing the same letter are significantly different ($P \leq 0.050$). Details of the statistical evaluations are shown in Table S9

myo-inositol-1(or 4)-monophosphatase in the $CaCO_3$ and $CaCO_3$ + formic acid treatments ($P \geq 0.269$) and decreased *myo*-inositol-1(or 4)-monophosphatase in the Ca-formate treatment ($P = 0.008$). *Myo*-inositol-1-phosphate synthase coding genes were lower in the $CaCO_3$ + formic acid and Ca-formate treatments than in the $CaCO_3$ treatment in the ileum ($P \leq 0.001$). Other genes annotated to InsP and *myo*-inositol degradation in the KEGG database were not influenced by the treatments used in this study.

$InsP_6$ disappearance and prececal digestibility of P and Ca

Contents of crop, gizzard, and ileum were analyzed for $InsP_6$ and degradation products, P, Ca, and titanium dioxide. $InsP_6$ disappearance and mineral digestibility were calculated using titanium dioxide as the undigestible reference. In the crop, Ca level did not affect $InsP_6$ disappearance ($P = 0.536$) when no phytase was supplemented (Fig. 7; Table S10). At the high Ca level, the effects of phytase supplementation on $InsP_6$ disappearance in the crop increased by 6 percentage points (**pp**) to 37% ($P = 0.025$). Phytase supplementation increased $InsP_6$ disappearance in the $CaCO_3$ treatments with and without formic acid by 20 and 37 pp., respectively ($P < 0.001$), but not in the Ca-formate treatment ($P = 0.090$).

Phytase supplementation increased pc $InsP_6$ disappearance ($P < 0.001$) to approximately 80%, irrespective of the dietary Ca level. Without phytase supplementation, pc $InsP_6$ disappearance was 9 pp. higher for the low compared to the high Ca level ($P < 0.001$). Ca level had no effect on pc $InsP_6$ disappearance for $CaCO_3$ + formic acid, but the high Ca level decreased pc $InsP_6$ disappearance by 4 pp. for $CaCO_3$ ($P = 0.047$) and by 10 pp. for Ca-formate ($P = 0.016$). Increasing dietary Ca decreased pc P digestibility. This effect was more pronounced for Ca-formate, with 12 pp. ($P < 0.001$), than for $CaCO_3$ and $CaCO_3$ + formic acid, with 7 pp. each ($P < 0.001$). The three-way interaction was significant for pc Ca digestibility ($P = 0.012$, Fig. 8). The pc Ca digestibility in $CaCO_3$ and $CaCO_3$-formate was increased by phytase supplementation at the low Ca level ($P \leq 0.012$) but was not affected at the high Ca level ($P \geq 0.160$). Phytase supplementation increased and decreased pc Ca digestibility for $CaCO_3$ + formic acid at the low and high Ca level, respectively ($P \leq 0.002$).

Inositol phosphate isomer and myo*-inositol concentrations*

Significant interactions between acidification and phytase supplementation were detected for $InsP_6$ and two $InsP_5$ isomers in the crop ($P < 0.001$; Table S11). Decrease in concentrations of $InsP_6$ and $InsP_5$ isomers upon phytase supplementation was most pronounced in the $CaCO_3$ + formic acid treatment and less in the $CaCO_3$ and the Ca-formate treatments. *Myo*-inositol concentrations in the crop were not affected.

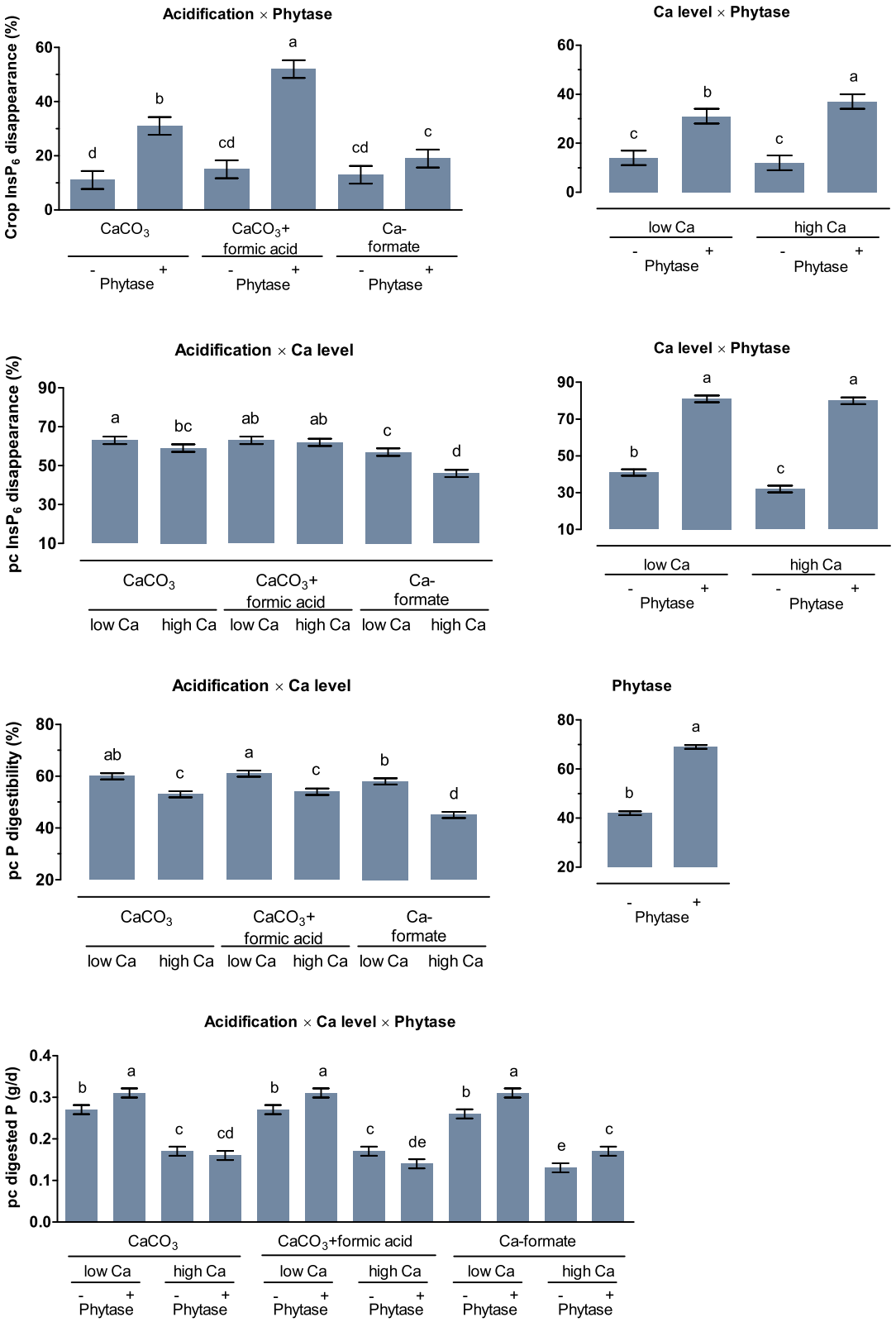

Fig. 7 (See legend on next page.)

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Fig. 7 $InsP_6$ disappearance in the crop, prececal (pc) $InsP_6$ disappearance, pc P digestibility, and amount of pc digested P of broiler chickens fed with differently acidified diets with low and high Ca levels without (–) and with (+) supplementation of 1500 FTU phytase/kg. Only significant ($P < 0.050$) effects are shown. Columns within a statistical comparison not sharing the same letter are significantly different ($P \leq 0.050$). Details of the statistical evaluations are shown in Table S10

In the gizzard, $InsP_6$ concentrations were lower in diets with supplemented phytase compared to those without ($P < 0.001$, Table S12). Highest $InsP_6$ concentrations were found in the Ca-formate treatment without phytase supplementation. Phytase supplementation decreased concentrations of $InsP_5$ isomers below level of detection, while making some $InsP_4$ and $InsP_3$ isomers detectable. Phytase supplementation increased *myo*-inositol concentrations to a greater extent at the low Ca level compared to the high Ca level ($P = 0.022$). Acidification had no effect on *myo*-inositol concentrations.

In the ileum, treatment effects on $InsP_6$ concentration were inverse to those on pc $InsP_6$ disappearance (Table S13). Phytase supplementation had no effect on Ins(1,2,4,5,6)P_5 concentration, but increased Ins(1,2,3,4,5)P_5 concentrations and decreased Ins(1,2,3,4,6)P_5 concentrations in most cases. Concentrations of $InsP_4$ and $InsP_{3x}$ were increased upon phytase supplementation ($P < 0.001$). Ins(1,2,3,4,5)P_5 concentrations were highest at the high Ca level for $CaCO_3$ + formic acid and both Ca levels for Ca-formate when phytase was supplemented. Concentrations of Ins(1,2,3,4)P_4 and $InsP_{3x}$ were high when phytase was supplemented to $CaCO_3$ + formic acid and Ca-formate at the high Ca level. An increase in *myo*-inositol concentrations upon phytase supplementation was more pronounced in the ileum at the low compared to the high Ca level. The highest *myo*-inositol concentration was determined for $CaCO_3$ + formic acid ($P \leq 0.003$) with no difference between $CaCO_3$ and Ca-formate ($P = 0.150$).

Discussion

Effects of phytase on $InsP_6$ degradation and subsequent P utilization differ considerably between studies. Possible explanations include the use of acidifying ingredients in the diet and different Ca levels affecting the pH,

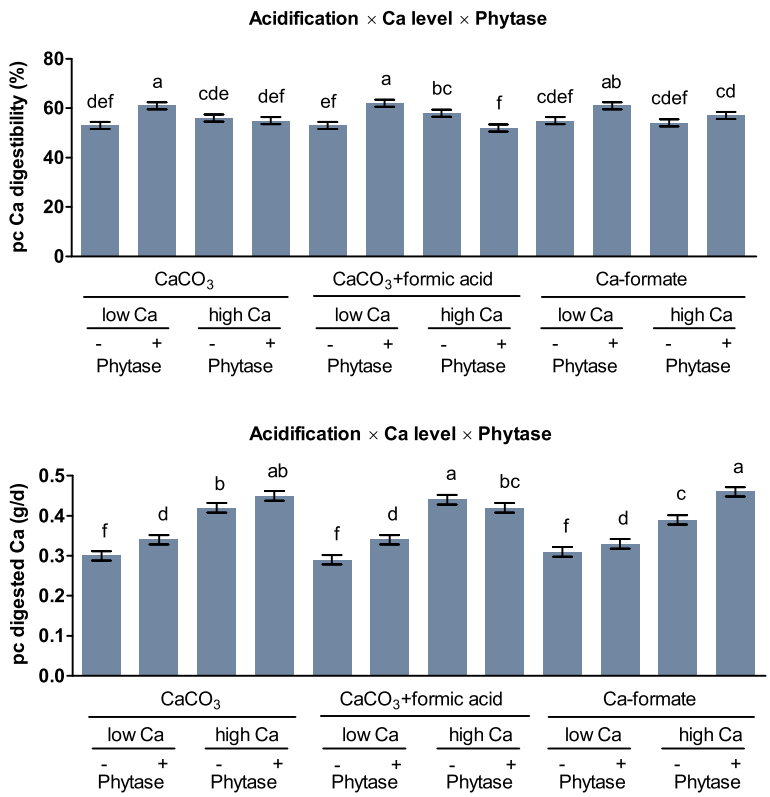

Fig. 8 Prececal (pc) Ca digestibility and amount of pc digested Ca of broiler chickens fed with differently acidified diets with low and high Ca levels without (–) and with (+) supplementation of 1500 FTU phytase/kg. Only significant ($P < 0.050$) effects are shown. Columns within a statistical comparison not sharing the same letter are significantly different ($P \leq 0.050$). Details of the statistical evaluations are shown in Table S10

phosphatase-producing bacteria, the probability that $InsP_6$ complexes are formed, and the efficacy of phytases in the digestive tract. Most studies only investigated one factor, making potential interactions impossible to detect. This study first-time investigated influences of phytase and acidifying ingredients with different Ca levels and connects effects on the microbial community and P utilization following $InsP_6$ degradation to find linkages between responses. We hypothesized that replacing $CaCO_3$ by Ca-formate or adding formic acid to $CaCO_3$-containing diets decreases the pH, influences the microbial community and its functionality, and increases $InsP_6$ degradation and P digestibility.

Crop

Crop microbiota and inositol phosphate degradation

In the crop, dietary treatments influenced both the microbial community and $InsP_6$ degradation. This could have been independent effects or causal linkages. Adding formic acid to $CaCO_3$ or replacing $CaCO_3$ with Ca-formate decreased pH in the crop content and concurrently shifted microbiota composition. A connection between pH and abundance of OTUs is indicated by correlations (Table S4) but it is not clear whether pH or other consequences of Ca level and acidification were causative. The microbial communities of the $CaCO_3$ + formic acid and Ca-formate treatments differed from that of the $CaCO_3$ treatment, while no difference of the microbial community was determined between the $CaCO_3$ + formic acid and Ca-formate treatments. Changes in pH were not completely concomitant with changes in the microbial community, suggesting that changes in the microbial community were caused by at least one other mechanism in addition to pH reduction. Likely, different mechanisms had an impact on the microbial community when the pH was reduced from 5.5 to 5.2 ($CaCO_3$ to Ca-formate) and from pH 5.2 to 4.9 (Ca-formate to $CaCO_3$ + formic acid). The microbial community may also have been affected by formic acid because the applied inclusion of formic acid is known to inhibit certain bacterial species including salmonella [13–15] and can be used as a carbon source by other bacteria [16].

Functional prediction and changes in the relative abundance of some bacteria may suggest an influence of the microbial community on P utilization. Phosphatase activity has been described for microorganisms, including *L. johnsonii* [17] and strains of *L. reuteri* [18]. Relative abundance of *L. johnsonii* ranged from 34 to 53% and depended on acidification and Ca level (Table S12). Hence, treatments probably influenced the contribution of *L. johnsonii* to P utilization and affected other traits of *L. johnsonii*; probiotic characteristics are assigned to *L. johnsonii*, such as reduced adhesion to the epithelial cells and inhibited growth of pathogenic microorganisms in humans [19], and they are further known to positively influence measures related to the immune system of broilers [20]. Relative abundance of OTUs assigned to strains of *L. reuteri* summed up to a range of 6–26%. At the low Ca level, the relative abundance of some OTUs assigned to *L. reuteri* (OTU4, OTU9, OTU11, and OTU13) were less abundant in the crop, which corroborates the finding that Ca can inhibit the growth of *L. reuteri* [18]. Hayek et al. [18] found that phosphatase activity (probably including phytase) produced by *L. reuteri* depended on strain and presence of chemical elements in a nutrient solution. Ca increased phosphatase activity of all *L. reuteri* strains under investigation. This effect was described to be caused by Ca-dependent activation of active sites of phosphatases, including phytase [21, 22]. In the present study, it appears possible that lower abundance of most *L. reuteri* strains were compensated by a higher potential to produce phosphatases by *L. reuteri* and other microorganisms at the higher Ca level when phytase was supplemented. Irrespective of individual bacteria strains, the InsP metabolism and other P-related metabolic pathways of the microbial community in the crop were influenced by acidification and Ca level. Within InsP metabolism, acidification and Ca level influenced the abundance of genes connected to enzymes involved in degradation of lower InsP isomers and *myo*-inositol. This strongly indicates that the microbial community contributes to InsP degradation in the crop; however, the extent of this contribution cannot be sufficiently quantified with the available data.

Effect of pH on $InsP_6$ disappearance in the crop

Acidification and Ca level effects were observed in diets with phytase supplementation only. With a value of 55%, the highest $InsP_6$ disappearance in the crop was observed when phytase was supplemented to $CaCO_3$ + formic acid. This effect seems to have been caused by acidification of the crop content by formic acid, which then shifted the pH closer to the optimum of the phytase used (pH optimum of 4.5 according to the product datasheet of the producer). This explanation is supported by a linearly negative relation between crop pH and $InsP_6$ disappearance in the phytase-supplemented diets (Fig. 9). The regression indicates that $InsP_6$ disappearance in the crop was increased by 5.3 pp. per each 0.1-unit reduction of pH in the crop.

Ca solubility effects

Different Ca solubility seems to have an influence on $InsP_6$ disappearance in the crop, but clear inferences are difficult to make. Higher solubility can make free Ca cations more available for Ca-$InsP_6$ complex

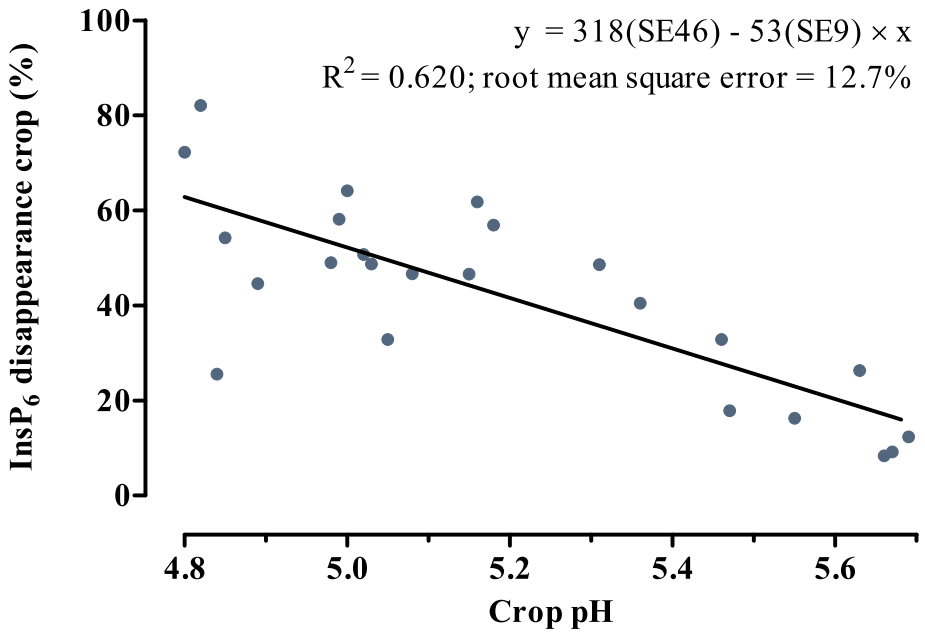

Fig. 9 Relationship between pH and $InsP_6$ disappearance in the crop of broiler chickens fed diets with $CaCO_3$ (without and with formic acid) at two dietary Ca levels with supplementation of 1500 FTU phytase/kg. Dots represent observations corrected for block effects

formation and, hence, reduce $InsP_6$ disappearance. Ca solubility of Ca-formate was reported to be substantially higher than that of $CaCO_3$ [23]. $InsP_6$ disappearance in the crop was higher for $CaCO_3$ compared to Ca-formate, consistent with the theory of higher Ca-$InsP_6$ complex formation as caused by higher solubility of Ca-formate. This is supported by the crop pH of 5.2 for Ca-formate and 5.5 for $CaCO_3$, both being above the pH critical for complex formation [9]. As acidification can increase Ca solubility, $CaCO_3$ + formic acid probably had higher Ca solubility than $CaCO_3$ alone as a consequence of formic acid lowering the pH [24]. Formic acid supplementation would then make more Ca available for Ca-$InsP_6$ complex formation and thus reduce $InsP_6$ disappearance. However, higher $InsP_6$ disappearance in the crop was observed for $CaCO_3$ + formic acid than for $CaCO_3$. When Ca-formate was used instead of $CaCO_3$, $InsP_6$ disappearance in the crop was possibly determined more by the higher Ca-$InsP_6$ complex formation caused by higher Ca solubility than by the lower pH. With formic acid supplementation, acidification may have had a higher impact on $InsP_6$ degradation than the higher Ca solubility.

Ileum and gizzard

Ileum microbiota and inositol phosphate degradation

The reducing effect of the high Ca level on pc $InsP_6$ disappearance for $CaCO_3$ but not for $CaCO_3$ + formic acid may be caused by changes in endogenous phytase and other phosphatases produced by epithelial cells or by microbiota. The latter is supported by the effects of the dietary treatments on metabolic pathways of the microbial community connected to $InsP_6$ degradation. Changes in P-related metabolic pathways in the ileum were similar to those in the crop. The composition of the microbial community varied among treatments. ANOVA analyses of relative abundances of single OTUs revealed significant interactions for five OTUs (1, 5, 7, 8, and 20; Table S5). However, an assessment of the contribution of these microorganisms to InsP degradation is difficult to derive because the phosphatase activity of the associated microorganisms is either not yet described in the literature or the OTUs define only classes of strains. Therefore, it cannot be estimated whether these microorganisms contributed phosphatase to a relevant extent. Nonetheless, results indicated that microorganisms in the digestive tract have contributed to P utilization of the birds. There were five positive correlations between pc P digestibility and OTUs assigned to *L. reuteri* and *L. johnsonii*; these microorganisms are known to produce phosphatase [17, 18]. Another possible explanation for differences in the microbial community could be an alteration of availability of nutrients for the microbiota in the digestive tract as a result of the varied supplements. To our knowledge, there is no literature available that supports or excludes one of these explanatory approaches.

Relation between calcium solubility, pH, and inositol phosphate degradation

High dietary Ca levels reduced pc $InsP_6$ degradation, but this effect was compensated by phytase supplementation. A decline in pc $InsP_6$ disappearance with graded levels of $CaCO_3$ in diets for broiler chickens was previously described [3]. In the present study, effects of acidification, Ca level, and phytase supplementation on $InsP_6$ degradation were interrelated. In contrast, Li et al. [7] and Dersjant-Li et al. [25] did not find an interaction between $CaCO_3$ level and phytase supplementation on pc $InsP_6$ disappearance and pc P digestibility when up to 1000 and 500 phytase units (**FTU**) phytase/kg were supplemented, respectively. However, dietary Ca levels in these studies were higher than in the present study and a different phytase product with different features such as pH optimum was used. We are not aware of other studies on $InsP_6$ degradation that compared $CaCO_3$ with the more soluble Ca-formate. Hamdi et al. [4] determined no significant difference in pc P digestibility when 5.5 g Ca/kg was supplied by $CaCO_3$ or the more soluble Ca chloride. In another series of experiments, $CaCO_3$ was compared with a calcified marine seaweed considered a highly soluble Ca source [26–28]. Results of this series were not consistent, likely owing to different Ca and phytase supplementation levels. Further, comparisons between calcified marine seaweed and Ca sources investigated in these studies may be impeded by unknown substances contained in the seaweed products. Overall, it seems that one major factor of P utilization affected by Ca sources and levels is the solubility of Ca from differing sources that depends, inter alia, on pH and microbiota. This supports conclusions drawn by Kim et al. [24] based on a comparison of $CaCO_3$ sources.

Differences in pc $InsP_6$ disappearance between $CaCO_3$, $CaCO_3$ + formic acid, and Ca-formate were smaller compared to $InsP_6$ disappearance in the crop. A clear determination of pc $InsP_6$ disappearance as compared to $InsP_6$ disappearance in the crop has been found in the present study, irrespective of whether phytase was supplemented or not (Fig. 10). This indicates the cleavage of Ca-$InsP_6$ complexes due to decreasing pH in the proventriculus and the gizzard. Gizzard pH ranged from 2.8–3.3, which was probably sufficient for cleavage of Ca-$InsP_6$ complexes because Ca-$InsP_6$ complexes occur at pH 4 and higher [29].

No difference in pc $InsP_6$ disappearance was found between $CaCO_3$ + formic acid at both Ca levels and the lower Ca level of $CaCO_3$. A reason for this could be the higher efficacy of phytase caused by a decreased crop pH and less complete $InsP_6$ degradation for Ca-formate due to higher Ca solubility. We are not aware of other studies investigating a simultaneous supplementation of formic acid and phytase in poultry. However, in pigs, total tract P digestibility was increased to a higher extent when phytase and

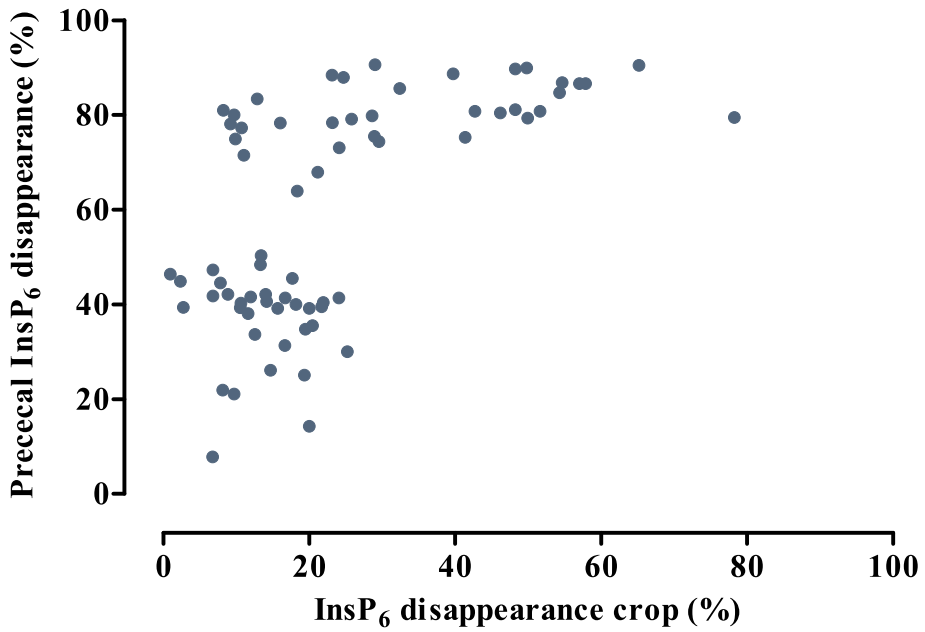

Fig. 10 Relationship between $InsP_6$ disappearance in the crop and prececal $InsP_6$ disappearance of broiler chickens. Dots represent observations corrected for block effects without (below 60% prececal InsP6 disappearance) or with (above 60% prececal $InsP_6$ disappearance) supplementation of 1500 FTU phytase/kg

formic acid were supplemented together than when their separate effects were summed [30].

Effects on inositol phosphate degradation pathways

Concentrations of lower InsP isomers in gastrointestinal content give insights into effects on InsP degradation pathways, including limiting steps in the process of InsP degradation. InsP isomers that limit InsP degradation apparently differed among treatments. When no phytase was supplemented, InsP degradation by endogenous enzymes seems to have been limited by the high Ca level. High Ca supposedly inhibited the activity of endogenous enzymes at different stages of the degradation pathway of $InsP_6$ and lower InsP isomers depending on the Ca source. This interpretation is based on lower concentrations of *myo*-inositol and higher concentrations of $InsP_6$ and $InsP_5$ isomers in the ileum at the high compared to the low Ca level of the $CaCO_3$ and Ca-formate treatments. In the $CaCO_3$ + formic acid treatment, there was no difference in $InsP_6$ concentrations in the ileum between the Ca levels, but there was slightly higher $InsP_5$ isomer and lower *myo*-inositol concentrations at the high Ca level. Obviously, formic acid supplementation overcame a restricted release of the first phosphate group from $InsP_6$ at the high Ca level that was observed in the $CaCO_3$ and Ca-formate treatments.

Degradation of lower InsP isomers in the ileum seems to be impaired by the high Ca level when phytase was supplemented. In the $CaCO_3$ treatment, higher $InsP_4$ and $InsP_3$ isomer concentrations and lower *myo*-inositol concentrations were found for the high compared to the low Ca level. This suggests a diminishing effect of a higher Ca level on phosphatases degrading $InsP_4$ and lower InsP isomers. Similar $InsP_3$ to $InsP_6$ concentrations were determined between the low Ca level of the $CaCO_3$ and the $CaCO_3$ + formic acid treatments, but higher *myo*-inositol concentrations were determined for the $CaCO_3$ + formic acid treatment. This may indicate a higher or faster hydrolysis of $InsP_2$ and $InsP_1$ isomers for the $CaCO_3$ + formic acid treatment at the low Ca level. A fast degradation of $InsP_2$ and $InsP_1$ isomers by the use of formic acid could also be observed in the higher Ca level. At high Ca levels, higher $InsP_5$ to $InsP_3$ concentrations were found for the $CaCO_3$ + formic acid treatment compared to the $CaCO_3$ treatment, but there was no difference in *myo*-inositol concentration. Despite a slower degradation of $InsP_5$ to $InsP_3$ isomers, this points towards a high hydrolysis of $InsP_2$ and $InsP_1$ isomers for the high Ca level for $CaCO_3$ + formic acid. However, it should be of greater interest to degrade InsP with higher phosphorylation first so as to diminish potential chelating effects. Comparing the low Ca levels in the $CaCO_3$ and Ca-formate treatments, higher concentrations of $InsP_6$ and $InsP_5$ isomers and similar *myo*-inositol concentrations were found, indicating that the initial steps of $InsP_6$ degradation were impeded for Ca-formate, and degradation of InsP isomers lower than $InsP_5$ was not the limiting factor. Degradation of $InsP_2$ and $InsP_1$ isomers seems to be particularly relevant for InsP degradation at both Ca levels for $CaCO_3$ + formic acid when phytase was supplemented. This makes the knowledge of $InsP_2$ and $InsP_1$ isomer concentrations necessary so as to determine InsP isomers that limit InsP degradation.

Effects on calcium digestibility

Responses in pc Ca digestibility differed from other traits under investigation, probably as a result of highly regulated Ca homeostasis and regulation of Ca absorption [31]. Phytase supplementation increased the pc digested amount of Ca irrespective of acidification at the low Ca level, whereas such an effect was only observed for Ca-formate at the high Ca level. This may be explained by a maximum pc digested Ca amount of about 0.42 g/d. This amount was met at the high Ca level without phytase supplementation for $CaCO_3$ and $CaCO_3$ + formic acid, but for Ca-formate only when phytase was supplemented. Particle size [28, 32] and Ca solubility [24] of Ca sources were shown to be a determinant of Ca utilization. Possibly, the considerably higher mean particle size of Ca-formate compared to $CaCO_3$ was more limiting for Ca utilization than the higher solubility of Ca-formate.

Conclusions

Replacing $CaCO_3$ by Ca-formate and adding formic acid to $CaCO_3$-containing diets decreased digesta pH, influenced the microbial community, and had an effect on $InsP_6$ degradation. Results imply that InsP degradation in the crop and until the terminal ileum may partly be explained by the microbial community because relationships between InsP degradation and relative abundance of phosphatase-producing strains *L. johnsonii* and *L. reuteri* were observed. Functional prediction also suggested influences of the microbiota on InsP degradation. In addition to microbiota, Ca effects on InsP degradation and pc P digestibility were shown to depend on dietary concentration, solubility of the Ca sources, as well as on the consequences of Ca supply on the pH of the digestive tract. The results of this study are relevant to the industry because choice of concentration and source of Ca in the diet is an important factor of feed formulation that affects P utilization by the animals.

Methods

Animals and management

The experiment was conducted at the Agriculture Experiment Station of the University of Hohenheim.

Unsexed Ross 308 broilers were obtained from a commercial hatchery (Brüterei Süd ZN der BWE-Brüterei Weser-Ems GmbH & Co. KG, Regenstauf, Germany). The hatchlings were placed in 72 floor pens (115 × 230 cm ground area, 260 cm height) in groups of 15. The temperature was gradually reduced from 34 °C at placement to 26 °C at the end of the experiment on day 22. The light regimen was 24:0 h of light:dark in the first three days and 18:6 h of light:dark from day 4 until the end of the experiment. Birds were kept on wood shavings for the first 15 days. On day 16, the litter was removed from the floor and birds were kept on perforated floors thereafter. Birds were reallocated among pens on day 16 to achieve a similar group weight in all pens. The pens were randomly assigned to the treatments in a completely randomized block design. Feed and water were available for ad libitum consumption throughout the experiment.

Diets

A commercial starter diet was provided for the first 15 days and contained, per kg, 215 g crude protein, 11 g Ca, 7 g P, 12.5 MJ metabolizable energy, 110 mg monensin sodium, 10 IU endo-1,4-β-xylanase, and 750 FTU of a 6-phytase (Deutsche Tiernahrung Cremer GmbH & Co. KG, Düsseldorf, Germany). From day 16, the experimental diets were provided.

Twelve experimental diets were mixed (Table S14). Except for Ca and P, the diets were calculated to meet or exceed the supply recommendations of the Gesellschaft für Ernährungsphysiologie [33]. The diets were based on corn, soybean meal, rapeseed meal, and sunflower meal and were formulated without mineral P. High levels of oilseed meals were included to achieve high $InsP_6$ concentrations as a substrate for the added phytase. Titanium dioxide was included as an indigestible marker at a level of 5 g/kg. Diets contained $CaCO_3$, $CaCO_3$ + formic acid (6 g/kg; Amasil 85, BASF SE, Germany; > 85% wt/wt formic acid), or Ca-formate. $CaCO_3$ and Ca-formate were added in two concentrations in order to achieve dietary Ca levels of 5.6 g Ca/kg dry matter ("low") or 8.2 g Ca/kg dry matter ("high"). One half of each diet was supplemented with 1500 FTU phytase/kg ("+"; Natuphos E 5000 G, BASF SE, Germany). The other half remained without phytase supplement ("-"). Diamol (diatomaceous earth) was used to balance mass differences between diets. Overall, analyzed nutrient concentrations confirmed calculated values (Table S15). The diets were produced by Research Diet Services (Research Diet Services BV, Hoge Maat 10, 3961NC, Wijk bij Duurstede, Netherlands) and pelleted through a 3-mm die.

Measurements and sampling procedures

Animals and feed were weighed on day 16 and at the end of the experimental period on a pen basis. At the end of the experiment, half of the pens of each treatment were processed on day 21 and day 22, respectively, for capacity reasons. The animals were deprived of feed 2 h before slaughter, and, feeders were moved back into the pens 1 h before slaughter in order to standardize gut fill. The animals were stunned using a gas mixture (35% CO_2, 35% N_2, and 30% O_2) and euthanized by CO_2 exposure. Crop and gizzard content was removed with a spatula without scraping the mucosa and pooled on a pen basis. A subsample of the pooled crop digesta was used for microbiota analysis and pH measurement. The section between Meckel's diverticulum and 2 cm anterior to the ileo-ceco-colonic junction, herein defined as the ileum, was removed. Digesta from the posterior half of the ileum was sampled because P absorption in the anterior third of the ileum may not have been completed [34]. Approximately 2 cm of this ileum section was used for pH and microbiota analysis. For this purpose, the digesta was carefully stripped out. Samples for microbiota analyses were immediately stored at − 20 °C. The digesta of the remainder of this ileum section was flushed out using ice-cold deionized water and pooled on a pen basis. Digesta samples were immediately frozen at − 20 °C until they were freeze-dried.

Microbial community analyses

DNA from crop and ileum digesta samples were extracted with the commercial DNA extraction kit FastDNA™ Spin Kit for soil (MP Biomedicals LLC, Solon, OH, USA). DNA was further quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA) and stored at − 20 °C. Illumina library was prepared according to Kaewtapee et al. [35]. The V1–2 region of the 16S rRNA gene was amplified and 1 μl of the first polymerase-chain reaction (**PCR**) product was used as a template in the second PCR with multiplexing and indexing primers as described previously [36]. Samples were sent for pair-end sequencing using the 250 bp paired-end sequencing chemistry on an Illumina MiSeq platform. Raw reads were checked for quality, assembled, and aligned using the mothur pipeline tool [37]. The data included 74,662 ± 3399 sequences per sample. The UCHIME program included in the mothur pipeline was used to identify possible chimeras [38]. Reads were clustered at 97% identity into 681 OTUs. Only OTUs with an average abundance higher than 0.0001% and a sequence longer than 250 bp were considered for further analysis. The closest representative was manually identified using seqmatch from the Ribosomal Database Project [39]. Sequences were submitted to European

Nucleotide Archive under the accession number PRJEB38378.

Prediction of functionality was carried out with the R package Tax4Fun2 [40], which relied on the SILVA database [41] and used the KEGG hierarchy, comprising of gene catalogs from sequenced genomes [42], for the assignations. To assign functionality, the BIOM table was obtained from the mothur pipeline [43]. Genomes from 16S rRNA gene sequences identified in this study were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/home/genomes/) in order to produce a database specially made for the crop and ileum of chickens.

Chemical and physical analyses

Samples of all diets were pulverized using a vibrating cup mill (Pulverisette 9, Fritsch GmbH, Idar-Oberstein, Germany) for chemical analyses of gross energy, P, Ca, Ti, InsP isomers, and *myo*-inositol, or ground to pass through a 0.5 mm sieve (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany) for all other analyses. Digesta samples were pulverized using the same vibrating cup mill. Ground samples were analyzed for proximate nutrients and fiber fractions according to the methods of Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten [44]. The concentrations of Ti, P, and Ca in pulverized feed and digesta samples were analyzed using inductively coupled plasma-optical emission spectrometry following wet digestion [45]. $InsP_6$ and $InsP_{3–5}$ isomers were analyzed in pulverized feed and digesta samples according to methods described by Zeller et al. [45] with modifications noted by Sommerfeld et al. [1]. Separation of enantiomers is not possible using this methodology; therefore, we were unable to distinguish between the D- and L-forms. Some $InsP_3$ isomers could not be identified because standards were unavailable. Clear discrimination of the isomers Ins (1,2,6) P_3, Ins (1,4,5) P_3, and Ins (2,4,5) P_3 was not possible because they co-eluted, and therefore the term $InsP_{3x}$ was used for these $InsP_3$ isomers of unknown proportions. *Myo*-inositol in feed and digesta samples was analyzed according to Sommerfeld et al. [46], using gas chromatography/mass spectrometry following derivatization. Phytase activity of the diets was analyzed according to an ISO standard method [47]. Measurements of pH in the undiluted content of the digestive tract were done using a CG 840 digital pH meter (Schott-Geräte GmbH, Mainz, Germany) equipped with a temperature probe and a puncture solid-state pH electrode suitable for semi-solid samples (InLab Solids Pro-ISM, Mettler Toledo Inc., Columbus, USA) as described previously [48].

Particle size distribution of $CaCO_3$ and Ca-formate were analyzed at Forschungsinstitut Futtermitteltechnik e.V. Braunschweig, Germany by laser diffraction (Sensor: HELOS Hi202, measuring range: 0.5/0.9–175 μm, dispersing system: Rodos/L (402F); SYMPATEC, Clausthal-Zellerfeld). Particle sizes of the diets were measured as described by Grubješić et al. [49] by wet sieving analysis using a sieve shaker (AS200, Retsch GmbH, Germany) with sieve sizes of 2, 1.18, 1, 0.5, 0.25, 0.125, and 0.063 mm.

Calculations and statistics

The ADG, ADFI, and G:F were calculated on a pen basis from day 16 to the end of the experiment and corrected for mortality. Prececal $InsP_6$ disappearance and pc digestibility of P and Ca (y) were calculated on a pen basis using the following equation:

$$\mathrm{y}(\%) = 100 - 100 \times \left(\frac{TiO_2 infeed(g/kgdrymatter)}{TiO_2 indigesta(g/kgdrymatter)} \times \frac{Analyteindigesta(g/kgdrymatter)}{Analyteinfeed(g/kgdrymatter)}\right) \quad (1)$$

Statistical evaluation of all traits was performed according to the following model:

$$\begin{aligned} \mathrm{y}_{ijkl} = {} & \mu + \mathrm{Acidification}_i + \mathrm{Calevel}_j + \mathrm{Phytase}_k \\ & + (\mathrm{Acidification}_i \times \mathrm{Calevel}_j) \\ & + (\mathrm{Acidification}_i \times \mathrm{Phytase}_k) \\ & + (\mathrm{Calevel}_j \times \mathrm{Phytase}_k) \\ & + (\mathrm{Acidification}_i \times \mathrm{Calevel}_j \times \mathrm{Phytase}_k) \\ & + \mathrm{block}_l + \mathrm{e}_{ijkl} \end{aligned} \quad (2)$$

where y_{ijkl} is the mean value of each treatment; μ is the mean of all treatments; $Acidification_i$ is the fixed effect of diets containing $CaCO_3$, $CaCO_3$ + formic acid, or Ca-formate; $Ca\ level_j$ is the fixed effect of the Ca level (5.6 or 8.2 g/kg dry matter); $Phytase_k$ is the fixed effect of phytase supplementation (0 or 1500 FTU phytase/kg); $block_l$ is the random block effect; and e_{ijkl} is the residual error. The block effect included possible effects of location in the building and sampling time on day 21 or day 22 because three blocks were sampled each day. ANOVA was calculated using the MIXED procedure of the SAS for Windows (version 9.4, SAS Institute, Cary, NC, USA). Normal distribution and homogeneity of variance were tested prior to statistical analysis. *P* values described herein result from ANOVA, or *t* tests when two groups were compared.

In order to describe the particle size distribution of the diets, the equation described by Siegert et al. [50] was fitted to the results of the sieve analysis using the NLMIXED procedure of SAS:

$$y = \frac{100}{1 + e^{(-a \times (x-b))}} \quad (3)$$

where y is the cumulative percentage of particles smaller than the sieve size x (mm), a is the slope of the regression, and b is the inflection point, which can be interpreted as the mean particle size (mm).

The sequencing dataset was statistically analyzed as described by Borda-Molina et al. [51] using the PRIMER software (PRIMER-E, version 7.0.9, Plymouth Marine Laboratory, Plymouth, UK) [52]. The dataset was first standardized by the total, then comparisons between samples were made through a sample similarity matrix using the Bray-Curtis coefficient algorithm. A hierarchical cluster analysis was done to show the similarity between samples. PERMANOVA was used to compare the microbial community among the treatments and between the sections (PRIMER-E, version 7.0.9, Plymouth Marine Laboratory, Plymouth, UK). The similarity percentage analysis (**SIMPER**) identified the OTU contribution to the similarity among samples within each treatment. Differences in the relative abundance of single OTUs were also estimated based on eq. 2. Pearson correlations with other traits were calculated for OTUs with a relative abundance > 1% using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Significance was declared at $P \leq 0.050$ for all statistical analyses.

Abbreviations

ADFI: Average daily feed intake; ADG: Average daily gain; ANOVA: Analysis of variance; Ca: Calcium; $CaCO_3$: Calcium carbonate; FTU: Phytase units; G:F: Gain to feed ratio; InsP: Inositol phosphate; $InsP_6$: *myo*-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); OTU: Operative taxonomic units; P: Phosphorus; pc: Prececal; PCR: Polymerase-chain; PERM ANOVA: Permutational multivariate analysis of variance; pp.: Percentage point; SIMPER: Similarity percentage analysis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-021-00083-7.

Additional file 1: Table S1. Growth performance. **Table S2.** Crop content and ileum digesta pH. **Table S3.** PERMANOVA of the microbial community in crop content and ileum digesta. **Table S4.** Relative abundance of OTUs in the crop content. **Table S5.** Relative abundance of OTUs in the ileum digesta. **Table S6.** Correlation between OTUs in the crop content and other measured traits. **Table S7.** Correlation between OTUs in the ileum content and other measured traits. **Table S8.** Influences on genes assigned to P-related pathways in crop content and ileum digesta. **Table S9.** Influences on genes assigned to enzymes related to inositol phosphate and *myo*-inositol degradation in crop content and ileum digesta. **Table S10.** $InsP_6$ disappearance and prececal P digestibility. **Table S11.** $InsP_6$, lower inositol phosphate isomers, and *myo*-inositol in the crop content. **Table S12.** $InsP_6$, lower inositol phosphate isomers, and *myo*-inositol in the gizzard digesta. **Table S13.** $InsP_6$, lower inositol phosphate isomers, and *myo*-inositol in the ileum digesta. **Table S14.** Composition of the experimental diets. **Table S15.** Analyses of experimental diets. **Figure S1.** Cluster analysis similarity in crop content and ileum digesta. **Figure S2.** Relationship crop pH and relative abundance of OTU2 in the crop content. **Figure S3.** Relation between the relative abundances of OTU1 and OTU2 with other measured traits.

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Further information

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Authors' contributions

JK, WS, VS, DF, and MR conceived and designed the experiment; JK, WS, DBM, VS, YPC, and HRT performed the experiments; JK, WS, and YPC conducted the animal data analysis; DBM, JK, WS, and ACS performed the microbial data analysis; JK, WS, and DBM conducted the statistical analysis; JK, WS, VS, and DBM drafted the paper; all authors revised the paper and approve of the final draft.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

The experiment was conducted in accordance with German Animal Welfare Legislation following approval of the Regierungspräsidium Tübingen, Germany (approval no. HOH53-18TE).

Consent for publication

Not applicable.

Competing interests

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

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