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# Short-chain fatty acids and ceca microbiota profiles in broilers and turkeys in response to diets supplemented with phytase at varying concentrations, with or without xylanase

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**ABSTRACT** Two independent studies were performed, each with a 3 × 2 factorial arrangement to compare the response in broilers and turkeys to phytase and xylanase supplementation on cecal fermentation and microbial populations. For both studies, 960 Ross 308 and 960 BUT 10 (1-day-old) were allocated to 1 of 6 experimental treatments: (1) control diet, containing the standard dose (100 g/ton) of phytase (**STD-Xyl**); (2) the control diet with 100 g/ton of xylanase (**STD + Xyl**); (3) the control diet supplemented on top with 2 fold the standard dose of phytase (200 g/ton), also referred as superdosing (**SD-Xyl**); (4) the superdosed diet with 100 g/ton of xylanase (**SD + Xyl**); (5) the control diet supplemented with 5-fold the standard dose of phytase (500 g/ton), also referred as megadosing (**MD-Xyl**); and (6) the megadosed diet with 100 g/ton of xylanase (**MD + Xyl**). Each treatment had 8 replicates of 20 animals. Broiler and turkey diets, based on wheat, soybean meal, rapeseed, and barley, and water were available ad libitum. On day 28, the cecal contents from 5 birds per pen were collected. The profile of short-chain fatty acids (**SCFA**) and microbiome structure (by % guanidine and cytosine [**G + C**] method) were analyzed. Selected % G + C fractions were used for 16S rDNA sequencing for the identification of bacteria. No treatment effects were noted on SCFA concentrations in either broilers or turkeys. Broilers fed MD diets had greater proportions of unclassified *Clostridiales*, *Mollicutes* (RF9) and *Faecalibacterium*. Xylanase supplementation in broilers resulted in lower proportions of *Lactobacillus* but increased *Mollicutes* (RF9), unclassified *Ruminococcus*, unclassified *Clostridiales*, and *Bifidobacterium*. The microbiome in turkeys was unaffected by phytase supplementation, but xylanase supplementation increased the proportions of *Lachnospiraceae* (*Incertae sedis*), *Lactobacillus*, and *Bifidobacterium*. Supplementation of turkey diets with increasing doses of phytase did not affect the cecal microbiota in contrast to what was observed in broilers. In contrast, xylanase supplementation in both species led to significant changes in the microbial populations, suggesting a positive influence through the provision of oligosaccharides.

**Key words:** exogenous enzymes, short-chain fatty acids, microbiota, broiler, turkey

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## INTRODUCTION

Exogenous enzymes to date have targeted host animal nutrition by destruction of antinutrients, increasing nutrient digestibility and hence improving animal performance (Choct and Annison, 1990). However, it has been known for more than 20 yr and recently re-emphasized that through their actions, nonstarch polysaccharide degrading enzymes produce nutrients for specific beneficial populations of bacteria, indicating that they are multifactorial in their effects (Bedford and Cowieson, 2012). For example, xylanase ameliorates the antinutritive effects of nonstarch polysaccharides by degrading soluble arabinoxylans, thus reducing digesta viscosity and improving nutrient digestibility (Bedford, 2002), but they have also been shown to release xylo-oligosaccharides (**XOS**) as a result of xylan degradation (Morgan et al., 2017). The presence of XOS in the distal sections of the gastrointestinal tract (**GIT**) may have a positive effect on the host as these molecules act as prebiotics or as signaling molecules for specific groups of



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beneficial bacteria influencing the production of short-chain fatty acids (**SCFA**) (Samanta et al., 2015; Bedford, 2018). As hypothesized De Maesschalck et al. (2015), the positive outcomes observed by XOS supplementation in broilers may be because of the direct stimulation of lactate producing bacteria, lactate being further fermented to butyrate in the large intestine. Thus, the beneficial effects resulting from inclusion of xylanases in poultry diets could result from the production of XOS and butyrate in addition to their direct activity on the soluble and viscous arabinoxylans.

Phytase can reduce the antinutritional effect of phytate catalyzing the stepwise hydrolysis of inositol phosphate esters (**InsPs**) and *myo*-inositol. As a result, phytase improves the digestibility of phosphorous (**P**), calcium (**Ca**), amino acids, and energy, as well as reduces excretion of inorganic P into the environment (Humer et al., 2015). Structural and functional changes of the microbiota in the GIT of broilers in response to phytase addition have also been shown (Ptak et al., 2015; Borda-Molina et al., 2016), possibly through alteration of nutrient flow. The combination of higher concentrations of phytase with xylanase in broiler diets has been shown to improve broiler performance (dos Santos et al., 2017), but comparatively little is known about the effects of phytase and xylanase supplementation in turkey rations. In presented the broiler and turkey, comparative response to phytase and xylanase supplementation on performance, nutrient digestibility, and ileal phytate degradation. This article aims to explore the response to phytase and xylanase in the cecal fermentation and the microbiota populations structure in broilers and turkeys from Olukosi et al. (2020).

## MATERIALS AND METHODS

### Birds and Housing

All the animal experiment procedures in the current studies were approved by Scotland's Rural College's Animal Experiment Committee.

A total of 960 each of Ross 308 male broilers and BUT 10 turkey poults were used in the 2 experiments. Upon arrival, birds were placed immediately in 48 floor pens with white pine shaving in environmentally controlled rooms, with 20 birds per pen on day 0. Each pen for broilers was 2.1 $m^2$ in size, whereas the pens for turkeys were 1.7 $m^2$. All pens were equipped with a hopper feeder and a bell drinker. Test diets and water were provided *ad libitum* throughout the trials. The rooms were preheated to 36°C 2 D before the commencement of the studies and kept at 36°C for the first 2 D. Then rooms temperature was gradually reduced to 23°C on day 21 (25°C for turkey) and were kept at 22°C (23°C–24°C for turkeys) until the end of the trials. From day 1, the dark hours were decreased daily by 1 h from 24 h light until the light–dark cycle were 18 h light and 6 h dark daily.

### Experimental Diets

Wheat, soybean meal, rapeseed, and barley were used as primary ingredients to formulate the experimental diets that met breeder recommendations for Ross 308 broilers and BUT 10 turkeys fed in 1 phase from 0 to 28 D of age. The compositions of the experimental diets and the analyzed chemical composition are shown in Table 1 and Table 2, respectively. For each animal species, 1 basal diet was made, then split equally into 6 subsamples each of which were supplemented with the experimental products: (1) control diet, containing the standard dose (100 g/ton) of phytase (Quantum Blue 5G; AB Vista, Marlborough, UK; 5,000 FTU/g) without xylanase (**STD-Xyl**); (2) the control diet with 100 g/ton of xylanase (Econase XT 25P; AB Vista, Marlborough, UK; 160,000 BXU/g) (**STD + Xyl**); (3) the control diet supplemented with 2-fold the standard dose of phytase (200 g/ton), referred to as superdosing (1,500 FTU/kg), without xylanase (**SD-Xyl**); (4) the superdosed diet with 100 g/ton of xylanase (**SD + Xyl**); (5) the control diet supplemented with 5-fold the standard dose of phytase (500 g/ton), referred to as megadosing (3,000 FTU/kg), without xylanase (**MD-Xyl**); and (6) the megadosed diet with

**Table 1.** Ingredient and calculated composition (%) of the experimental basal diets.

| Items | Broiler | Turkey |
|---|---|---|
| Wheat | 62.53 | 50.00 |
| Barley | 5.00 | 5.00 |
| Soybean meal | 17.62 | 26.34 |
| Rapeseed meal | 10.00 | 11.77 |
| Soya oil | 0.50 | 1.03 |
| Salt | 0.22 | 0.18 |
| Limestone | 0.66 | 0.79 |
| Dicalcium phosphate | 1.01 | 2.45 |
| Sodium bicarbonate | 0.10 | 0.10 |
| L-Tryptophan | 0.05 | 0.00 |
| Lysine HCl | 0.55 | 0.65 |
| DL-Methionine | 0.33 | 0.33 |
| L-Threonine | 0.23 | 0.19 |
| L-Valine | 0.19 | 0.12 |
| Trace mineral-vitamin premix[1] | 0.50 | 0.50 |
| Quantum Blue 5G[2] | 0.01 | 0.01 |
| Titanium oxide | 0.5 | 0.5 |
| Total | 100 | 100 |
| Calculated composition (% as feed) | | |
| AME, kcal/kg | 2,800 | 2,700 |
| Crude protein | 20.52 | 24.00 |
| Ca | 0.90 | 1.34 |
| Available P | 0.31 | 0.60 |
| Fat | 2.13 | 2.65 |
| Crude fiber | 3.33 | 3.58 |
| D Met + Cys | 0.92 | 1.01 |
| D Lys | 1.25 | 1.56 |
| D Trp | 0.26 | 0.25 |
| D Thr | 0.82 | 0.91 |
| D Val | 0.95 | 1.04 |

[1]Vitamin/mineral premix supply per kilogram of diet: vitamin A, 16,000 IU; vitamin $D_3$, 3,000 IU; vitamin E, 25 IU; vitamin $B_1$, 3 mg; vitamin $B_2$, 10 mg; vitamin $B_6$, 3 mg; vitamin $B_{12}$, 15 µg; nicotinic acid, 60 mg; pantothenic acid, 14.7 mg; folic acid, 1.5 mg; biotin, 125 µg; choline chloride, 25 mg; Fe as iron sulfate, 20 mg; Cu as copper sulfate, 10 mg; Mn as manganese oxide, 100 mg; Co as cobalt oxide, 1.0 mg; Zn as zinc oxide, 82.222 mg; I as potassium iodide, 1 mg; Se as sodium selenite, 0.2 mg; and Mo as molybdenum oxide, 0.5 mg.

[2]Quantum Blue 5G, AB Vista, Marlborough, UK; 5,000 FTU/g.

**Table 2.** Analyzed composition (%, as fed) of the experimental diets.

| Treatment | DM | N | Ca | Na | Mg | Cu, ppm | Fe, pp, | Mn, ppm | Zn, ppm | K | P | InsP6, nmol/g |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Broiler | | | | | | | | | | | | |
| 1 | 89.3 | 3.37 | 0.74 | 0.11 | 0.16 | 16 | 122 | 115 | 98 | 0.86 | 0.54 | 13,733 |
| 2 | 89.2 | 3.20 | 0.74 | 0.11 | 0.16 | 14 | 125 | 123 | 104 | 0.80 | 0.52 | 15,570 |
| 3 | 89.6 | 3.28 | 0.79 | 0.12 | 0.17 | 14 | 149 | 126 | 109 | 0.83 | 0.58 | 13,183 |
| 4 | 89.5 | 3.29 | 0.80 | 0.13 | 0.18 | 16 | 136 | 124 | 102 | 0.89 | 0.58 | 13,639 |
| 5 | 89.0 | 3.24 | 0.70 | 0.11 | 0.16 | 15 | 121 | 119 | 96 | 0.79 | 0.53 | 13,605 |
| 6 | 89.2 | 3.14 | 0.78 | 0.13 | 0.16 | 18 | 134 | 124 | 104 | 0.81 | 0.56 | 10,581 |
| Turkey | | | | | | | | | | | | |
| 1 | 89.3 | 3.89 | 1.05 | 0.10 | 0.17 | 16 | 130 | 107 | 103 | 0.84 | 0.72 | 8,037 |
| 2 | 89.2 | 3.90 | 1.11 | 0.10 | 0.18 | 15 | 124 | 108 | 104 | 0.96 | 0.77 | 8,098 |
| 3 | 89.6 | 3.79 | 1.02 | 0.08 | 0.17 | 19 | 122 | 100 | 94 | 0.88 | 0.73 | 7,651 |
| 4 | 89.5 | 3.83 | 1.16 | 0.11 | 0.18 | 18 | 125 | 126 | 106 | 0.94 | 0.78 | 8,503 |
| 5 | 89.0 | 3.86 | 1.08 | 0.10 | 0.23 | 17 | 123 | 109 | 101 | 0.90 | 0.75 | 8,396 |
| 6 | 89.2 | 3.87 | 1.07 | 0.10 | 0.17 | 16 | 115 | 102 | 98 | 0.90 | 0.75 | 7,824 |

100 g/ton of xylanase (**MD + Xyl**) resulting in 6 experimental treatments (Table 3). Diets were presented in mash form. Experimental diets did not contain any coccidiostat, antibiotic, or any other growth promoter.

## Experimental Procedures

For both experiments, on day 28, 5 birds from each of the 48 floor pens were randomly selected and euthanized by cervical dislocation. The total GIT was removed immediately from the abdominal cavity. The digesta content from the ceca was immediately collected by gently squeezing each section into a tube and pooled per pen, rapidly frozen on dry ice, and stored at −20°C for subsequent analysis of SCFA and the microbial populations structure by the percentage of guanidine and cytosine (**G + C**) method.

## Sample Analyses

Feed samples were analyzed for dry matter, nitrogen, and minerals. The analyses were performed according to (AOAC, 2006) Official Methods. Dry matter was determined by drying the samples in a drying oven (Uniterm, Russel-Lindsey Engineering Ltd., Birmingham, England, UK) at 100°C for 24 h (AOAC Method 934.01). Total nitrogen content was determined by the Dumas combustion method (Method 968.06). Mineral content was determined using inductively coupled plasma–optical emission spectroscopy following digestion, in turn, in concentrated $HNO_3$ and HCl.

The SCFA were analyzed as free acids by gas chromatography, using pivalic acid as an internal standard (Apajalahti et al., 2019). Briefly, 1 mL of $H_2O$ was mixed with 1 g of ceca content, and then 1 mL of 20 mmol/L pivalic acid solution was added as an internal standard. After mixing, 1 mL of perchloric acid was added, and SCFA were extracted by shaking the mixture for 5 min. After centrifugation, perchloric acid in the supernatant was precipitated by adding 50 µL of 4 mol KOH in 500 µL of supernatant. After 5 min, saturated oxalic acid was added, and the mixture incubated at 4°C for 60 min and then centrifuged at 18,000 × *g* for 10 min. Samples were analyzed by gas chromatography using a glass column packed with 80/120 Carbopack B-DA/4% Carbowax 20 mol stationary phase (Supelco, Bellefonte, PA), using helium as the carrier gas and a flame ionization detector. The acids measured were acetic, propionic, butyric, iso-butyric, 2-methyl-butyric, iso-valeric, and lactic acid.

Cecal digesta samples pooled from 5 birds per pen were collected, and bacterial DNA was extracted as previously described (Apajalahti et al., 1998). The DNA samples were then fractionated by 72 h CsCl equilibrium density gradient ultracentrifugation (100,000 × *g*), which separates chromosomes with different G + C

**Table 3.** Analyzed enzyme activities in feed samples.

| Treatments[1] | | | | Broiler | | Turkey | |
|---|---|---|---|---|---|---|---|
| Number | Identification | Phytase (FTU/kg) | Xylanase (BXU/kg) | Phytase[2] (FTU/kg) | Xylanase[3](BXU/kg) | Phytase (FTU/kg) | Xylanase (BXU/kg) |
| 1 | STD-Xyl | 500 | 0 | 750 | <2,000 | 954 | <2,000 |
| 2 | STD + Xyl | 500 | 16,000 | 865 | 16,100 | 994 | 16,100 |
| 3 | SD-Xyl | 1,500 | 0 | 1,190 | <2,000 | 2,880 | <2,000 |
| 4 | SD + Xyl | 1,500 | 16,000 | 2.250 | 14,300 | 1,840 | 15,000 |
| 5 | MD-Xyl | 3,000 | 0 | 2,720 | <2,000 | 3,790 | <2,000 |
| 6 | MD + Xyl | 3,000 | 16,000 | 3,930 | 17,400 | 2,460 | 13,300 |

[1]Diets consisted in 6 experimental treatments: STD-Xyl (diet containing standard dose of phytase without xylanase), STD + Xyl (diet containing standard dose of phytase with xylanase), SD-Xyl (diet containing superdosing of phytase without xylanase), SD + Xyl (diet containing superdosing with xylanase), MD-Xyl (diet containing megadosing of phytase without xylanase) and MD + Xyl (diet containing megadosing with xylanase).

[2]One FTU is defined as the amount of enzyme required to release 1 µmol of inorganic P per minute from sodium phytate at 37°C and pH 5.5.

[3]One BXU is defined as the amount of enzyme that produces 1 nmol reducing sugars from birchwood xylan in 1 s at 50°C and pH 5.3.

content (Apajalahti et al., 1998). This separation is based on differential density imposed by the adenine–thymine–dependent DNA-binding dye bisbenzimidazole. Following the ultracentrifugation, the formed gradients were pumped through a flow-through UV absorbance detector set to 280 nm, and % G + C fractions were collected at 5 to 7% intervals. Finally, the % G + C content represented by each gradient fraction was determined by linear regression analysis ($r^2 > 0.99$).

Fifteen pooled % G + C fractions were used for 16S rDNA sequencing. Altogether 15 DNA pools were subjected to desalting with PD-10 columns (GE Healthcare, UK) for subsequent 16S rRNA gene PCR amplification with the universal broad-range primer pair. The PCR products were sequenced with the Illumina MiSeq (Illumina, San Diego, CA) next-generation sequencing platform. Raw sequence data were subjected to standard next-generation sequencing data preprocessing and data analysis: demultiplexing of all libraries for each sequence lane using Illumina bcl2fastq 2.17.1.14 software (Illumina), sorting of reads by amplicon inline barcodes, clipping of the adapters, primer-based sorting, sequence alignment, combining of the forward and reverse reads using BBMerge 34.48, generating consensus sequences, and grouping. 16S rRNA gene data were processed, and operational taxonomic units (**OTU**) were picked from amplicons with Mothur 1.35.1 program: alignment was done against 16S rRNA SILVA SEED r119 reference alignment; short alignments (truncated or unspecific PCR products) and chimeras were filtered; sequences were taxonomically classified against the SILVA databases and sequences from other domains of life were removed; OTU were picked by clustering at the 97% identity level; and OTU consensuses were taxonomically classified to genus level.

Operational taxonomic units diversity analysis was performed with QIIME 1.9.0 (Caporaso et al., 2010): within-sample diversity was analyzed at minimum and median sample sequence count rarefaction levels ("alpha diversity"), including creation of plots and tables with taxonomical sample composition; between-sample diversity was analyzed at minimum and median sample sequence count rarefaction levels ("beta diversity").

### Statistical Analysis

The effect of treatments for SCFA for each study were subjected to 2-way analysis of variance using JMP 14 Pro (SAS). Additionally, the effect of bird species for SCFA were subjected to one-way analysis of variance. The effect of treatments for % G + C profile were subjected to *t*-test analysis. Pen was the experimental unit. Means were separated only when the treatment *P*-value was significant and then by using the least significant difference test. Statements of significance were based on *P*-value of equal to or less than 0.05.

## RESULTS

All growth performance, ileal digestibility, and digesta inositol phosphate concentration results from the experiments are presented in (Olukosi et al., 2020). The nutrient profile in the diets (Table 2) and the xylanase activities (Table 3) were met, but the analyzed phytase in the diets were greater than expected (Table 3).

### Cecal Fermentation

The effects of experimental treatments on the concentration of SCFA in the cecum of broilers and turkeys at 28 D of age are presented in Tables 4 and 5, respectively.

**Table 4.** Concentration of ceca short-chain fatty acids (SCFA) (mmol/L) in broilers measured at day 28.[1,2]

| Phytase, FTU/kg | Xylanase, BXU/kg | Acetic | Propionic | Butyric | Iso-butyric | 2-Me-butyric | Iso-valeric | Lactic | BCFAs[4] | VFAs[5] | Total SCFAs[6] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Main effects means for phytase | | | | | | | | | | | |
| 500 (STD) | | 90.69 | 4.39 | 22.61 | 1.00 | 0.21 | 0.31 | 9.19 | 1.52 | 119.19 | 128.63 |
| 1,500 (SD) | | 93.09 | 4.04 | 21.93 | 0.92 | 0.11 | 0.23 | 10.69 | 1.25 | 120.25 | 131.13 |
| 3,000 (MD) | | 94.10 | 4.01 | 23.40 | 0.91 | 0.08 | 0.21 | 9.94 | 1.19 | 122.75 | 132.63 |
| Least significant difference | | 8.56 | 0.78 | 4.26 | 0.12 | 0.26 | 0.22 | 4.91 | 0.48 | 11.22 | 13.26 |
| Main effect means for xylanase | | | | | | | | | | | |
| | 0 (−Xyl) | 91.89 | 4.08 | 22.28 | 0.97 | 0.15 | 0.29 | 10.04 | 1.41 | 119.63 | 129.88 |
| | 16,000 (+Xyl) | 93.37 | 4.22 | 23.01 | 0.91 | 0.10 | 0.20 | 9.83 | 1.23 | 121.83 | 131.71 |
| Least significant difference | | 6.99 | 0.63 | 3.48 | 0.10 | 0.21 | 0.18 | 4.01 | 0.39 | 9.16 | 10.82 |
| Standard deviation | | 11.99 | 1.09 | 5.97 | 0.16 | 0.36 | 0.31 | 6.88 | 0.68 | 15.73 | 18.58 |
| *P*-value for main effects and interaction[3] | | | | | | | | | | | |
| | Phytase | 0.719 | 0.552 | 0.778 | 0.217 | 0.549 | 0.613 | 0.825 | 0.332 | 0.808 | 0.832 |
| | Xylanase | 0.676 | 0.652 | 0.668 | 0.219 | 0.625 | 0.345 | 0.916 | 0.332 | 0.631 | 0.737 |
| Phytase × Xylanase | | 0.205 | 0.483 | 0.913 | 0.598 | 0.788 | 0.809 | 0.532 | 0.859 | 0.301 | 0.242 |

[1]Data are means of 5 birds per pen with 8 pens per treatment.
[2]Diets consisted in 6 experimental treatments: STD-Xyl (diet containing standard dose of phytase without xylanase), STD + Xyl (diet containing standard dose of phytase with xylanase), SD-Xyl (diet containing superdosing of phytase without xylanase), SD + Xyl (diet containing superdosing with xylanase), MD-Xyl (diet containing megadosing of phytase without xylanase) and MD + Xyl (diet containing megadosing with xylanase).
[3]Values in the same column with different letters are significantly different ($P < 0.05$).
[4]BCFA: branched-chain fatty acids (iso-butyric + 2-me-butyric + iso-valeric).
[5]VFA: volatile fatty acids (acetic acid + propionic acid + butyric acid + BCFA).
[6]Total SCFA: total short-chain fatty acids (VFA + lactic acid).

**Table 5.** Concentration of ceca short-chain fatty acids (SCFA) (mmol/L) in turkeys measured at day 28.[1,2]

| Phytase, FTU/kg | Xylanase, BXU/kg | Acetic | Propionic | Butyric | Iso-butyric | 2-Me-butyric | Iso-valeric | Lactic | BCFAs[4] | VFAs[5] | Total SCFAs[6] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Main effect means for phytase | | | | | | | | | | | |
| 500 (STD) | | 62.11 | 1.01 | 11.33 | 0.04 | 0.05 | 0.00 | 25.69 | 0.09 | 74.69 | 100.25 |
| 1,500 (SD) | | 63.78 | 1.28 | 11.83 | 0.12 | 0.03 | 0.03 | 23.70 | 0.17 | 77.04 | 100.88 |
| 3,000 (MD) | | 67.31 | 1.51 | 11.88 | 0.09 | 0.00 | 0.02 | 29.56 | 0.11 | 80.69 | 110.31 |
| Least significant difference | | 8.42 | 0.60 | 3.61 | 0.11 | 0.07 | 0.05 | 10.02 | 0.14 | 11.85 | 9.30 |
| Main effect means for xylanase | | | | | | | | | | | |
| | 0 (−Xyl) | 65.37 | 1.40 | 12.05 | 0.09 | 0.00 | 0.01 | 25.13 | 0.10 | 78.92 | 104.13 |
| | 16,000 (+Xyl) | 63.43 | 1.14 | 11.31 | 0.08 | 0.05 | 0.02 | 27.51 | 0.14 | 76.02 | 103.50 |
| Least significant difference | | 6.87 | 0.49 | 2.95 | 0.09 | 0.06 | 0.04 | 8.18 | 0.11 | 9.68 | 7.59 |
| Standard deviation | | 11.79 | 0.84 | 5.06 | 0.15 | 0.10 | 0.07 | 14.03 | 0.20 | 16.60 | 13.02 |
| *P*-value for main effects and interactions[3] | | | | | | | | | | | |
| | Phytase | 0.452 | 0.254 | 0.943 | 0.353 | 0.344 | 0.541 | 0.500 | 0.505 | 0.592 | 0.062 |
| | Xylanase | 0.577 | 0.310 | 0.623 | 0.733 | 0.069 | 0.759 | 0.564 | 0.496 | 0.554 | 0.870 |
| Phytase × Xylanase | | 0.862 | 0.139 | 0.922 | 0.465 | 0.344 | 0.203 | 0.093 | 0.428 | 0.838 | 0.166 |

[1]Data are means of 5 birds per pen with 8 pens per treatment.
[2]Diets consisted in 6 experimental treatments: STD-Xyl (diet containing standard dose of phytase without xylanase), STD + Xyl (diet containing standard dose of phytase with xylanase), SD-Xyl (diet containing superdosing of phytase without xylanase), SD + Xyl (diet containing superdosing with xylanase), MD-Xyl (diet containing megadosing of phytase without xylanase) and MD + Xyl (diet containing megadosing with xylanase).
[3]Values in the same column with different letters are significantly different ($P < 0.05$).
[4]BCFA: branched-chain fatty acids (iso-butyric + 2-me-butyric + iso-valeric).
[5]VFA: volatile fatty acids (acetic acid + propionic acid + butyric acid + BCFA).
[6]Total SCFA: total short-chain fatty acids (VFA + lactic acid).

There were no treatment effects observed for any of the SCFA measured in broilers or turkeys.

## Microbial Population Structure

The effects of experimental treatments on the percentage of G + C in the cecum of broilers and turkeys at 28 D of age are presented in Figure 1A and B, respectively. The regions between 28 and 29% G + C fraction ($P < 0.05$) and 73 to 75% G + C fraction ($P < 0.05$) in broiler chickens were significantly lesser in abundance in birds fed the standard dose of phytase compared with those fed the megadosed diet (Figure 1A). In turkeys, no differences were observed when all treatments were considered in the statistical analysis (Figure 1B).

Turkeys differed significantly from broilers with regards to density of population of microbes between 21 to 29, 50 to 61, 64 to 69, and 75% G + C fractions ($P < 0.05$) (data not shown). The identification of the microbiota profile behind these regions and the comparison between both bird species will be covered in the discussion section.

The effect of phytase on broiler microbiota was explored isolating only STD and MD treatments without xylanase (Figure 2A). MD phytase increased species between 21 and 29% G + C fraction, ($P < 0.05$) and the 60 to 63% G + C ($P \leq 0.10$). In contrast, the highest dose of phytase tended to decrease the numbers of bacteria found in the regions 39 to 41 and 50 to 53% G + C ($P \leq 0.10$). Bacteria behind these shifts were identified through 16S rDNA sequencing of the selected % G + C fractions. In % G + C fraction from 24 to 29%, unclassified *Ruminococcaceae*, *Clostridiales*, *Mollicutes* (RF9 group), unclassified *Bacteria*, and *Lactobacillus* were the most abundant bacterial genera in both treatments (Figure 2B). High doses of phytase increased the proportion unclassified *Clostridiales* by approximately 10% and the proportion of *Mollicutes* (RF9 group) by 4%. In % G + C fraction from 44 to 49%, *Lachnospiraceae* (*Incertae sedis*), unclassified *Lachnospiraceae*, and *Lactobacillus* were the most abundant bacteria (Figure 2C); however, proportions of the different bacteria did not differ with high doses of phytase when compared with the standard dose. In % G + C fraction from 64 to 69%, *Bifidobacterium* made up more than half of the total bacterial sequences in both treatments (Figure 2D). Megadosing reduced the proportion of *Bifidobacterium* by 5% units and increased the proportion *Faecalibacterium* by approximately 16% units.

The effect of xylanase in broiler microbiota was studied only in treatments having standard doses of phytase without and with xylanase (Figure 3A). Xylanase supplementation tended to increase the abundance of bacteria at % G + C 63 to 64 and to reduce the abundance of 32 to 37%, the latter being mainly attributed to *Lactobacillus*, *Mollicutes* (RF9 group), unclassified *Ruminococcus*, and unclassified *Clostridiales* (Figure 3B). Broilers receiving xylanase had 9% less *Lactobacillus* with a concomitant increase in the proportion of *Mollicutes* (RF9 group), unclassified *Ruminococcus*, and unclassified *Clostridiales*. Figure 3C illustrates the distribution of bacteria identified in the high % G + C fractions (64 to 69%) of the STD treatment with and without xylanase. *Bifidobacterium* was the most abundant genus. Broilers receiving xylanase-supplemented diet had greater proportion of bifidobacterial sequences by 15%.

The increasing doses of phytase did not result in any significant change in turkey's microbiota using % G + C profiling (data not shown). However, more interesting results were observed when xylanase was included in the diet regardless the dose of phytase. Figure 4A

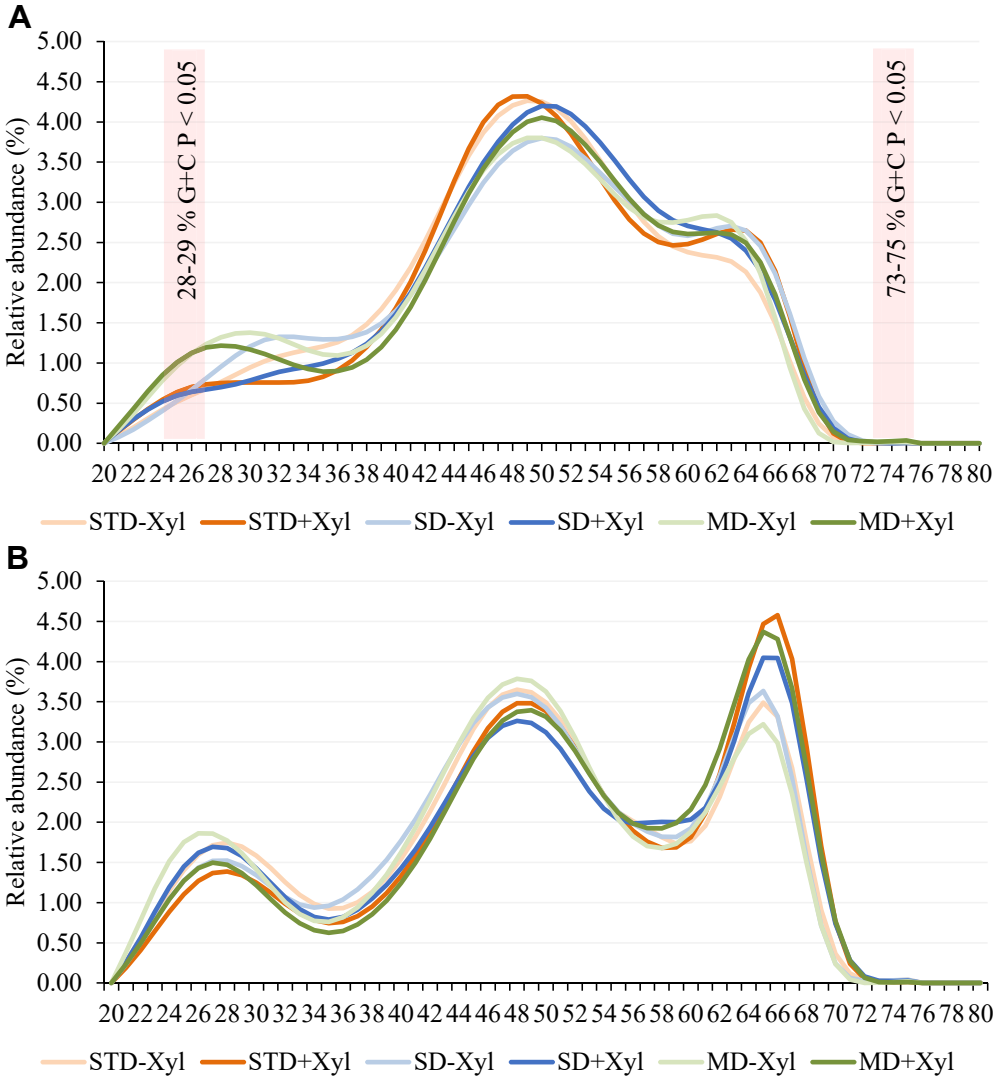


**Figure 1.** Effect of phytase and xylanase on cecal microbiota of broiler chickens (A) and turkeys (B) at 28 D of age. Diets consisted in 6 experimental treatments: STD-Xyl (diet containing standard dose of phytase without xylanase), STD + Xyl (diet containing standard dose of phytase with xylanase), SD-Xyl (diet containing superdosing of phytase without xylanase), SD + Xyl (diet containing superdosing with xylanase), MD-Xyl (diet containing megadosing of phytase without xylanase) and MD + Xyl (diet containing megadosing with xylanase). Data are means of 5 birds per pen with 8 pens per treatment. The colored regions indicates the % G + C profile which are statistically different ($P \leq 0.05$) between treatment groups by Student *t*-test.

shows the % G + C shifts of the cecal microbiota of turkeys without and with xylanase, when all phytase doses were combined. Xylanase reduced the abundance of bacteria representing the % G + C region 36 to 48 ($P < 0.05$) but increased the proportion of bacteria in the % G + C region from 64 to 69% ($P < 0.05$). Xylanase addition increased bacterial diversity in the % G + C from 44 to 49% (Figure 4B). *Lachnospiraceae* (*I. sedis*), unclassified *Lachnospiraceae*, *Anaerofilum*, *Faecalibacterium*, *Lactobacillus*, *Subdoligranulum*, and *Erysipelotruchaceae (I. sedis)* were the most dominant bacterial genera in both treatments. Xylanase supplementation increased proportion of *Lachnospiraceae* (*I. sedis*) by 8% and *Lactobacillus* by 2.5%, but the proportion of *Anaerofilum* was reduced by about 3% units. Finally, sequence identification of the % G + C region from 64 to 69% is presented in Figure 4C. Sequences of *Bifidobacterium* dominated this region. Supplementation with xylanase increased the proportion of *Bifidobacterium* by 13%.

## DISCUSSION

The aim of the current experiment was to explore the effects of phytase and xylanase in broilers and turkeys on the concentration of SCFA and the microbiome in the ceca. Diets were formulated to meet or exceed nutrient recommendations fed in 1 single phase from day 0 to day 28. As a result, the diets for both species were different because of their significantly different requirements. This introduces a source of variation which is likely significant to interpretation of the results for comparison purposes. Protein, Ca, and P were considerably greater in turkey compared with broiler diets. High Ca/P diets may induce greater buffering capacity which increases digesta pH and may result in undigested

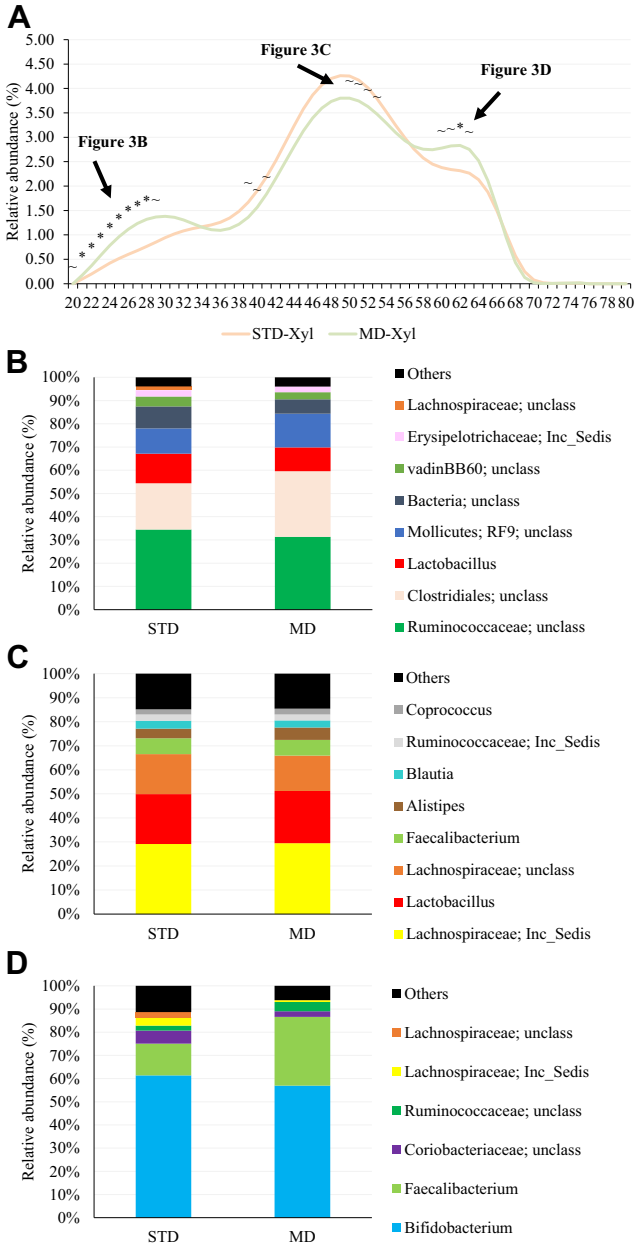


**Figure 2.** (A) Effect of high doses of phytase (MD) on cecal microbiota of broiler chickens compared to standard doses of phytase (STD) without xylanase at 28 D of age. Data are means of 5 birds per pen with 8 pens per treatment. The tildes (~) indicate the % G + C profile which are $P \leq 0.10 > 0.05$ and asterisks (*) indicate the % G + C profile which are statistically different ($P \leq 0.05$) by Student *t*-test. (B) Distribution of the bacterial genera identified in the low % G + C fraction (24–29%) in broilers fed standard doses of phytase (STD) and high doses of phytase (MD) without xylanase. (C) Distribution of bacterial general identified in the mid % G + C fraction (44–49%) in broilers fed standard doses of phytase (STD) and high doses of phytase (MD) without xylanase. (D) Distribution of bacterial general identified in the high % G + C fraction (64–69%) in broilers fed standard doses of phytase (STD) and high doses of phytase (MD) without xylanase.

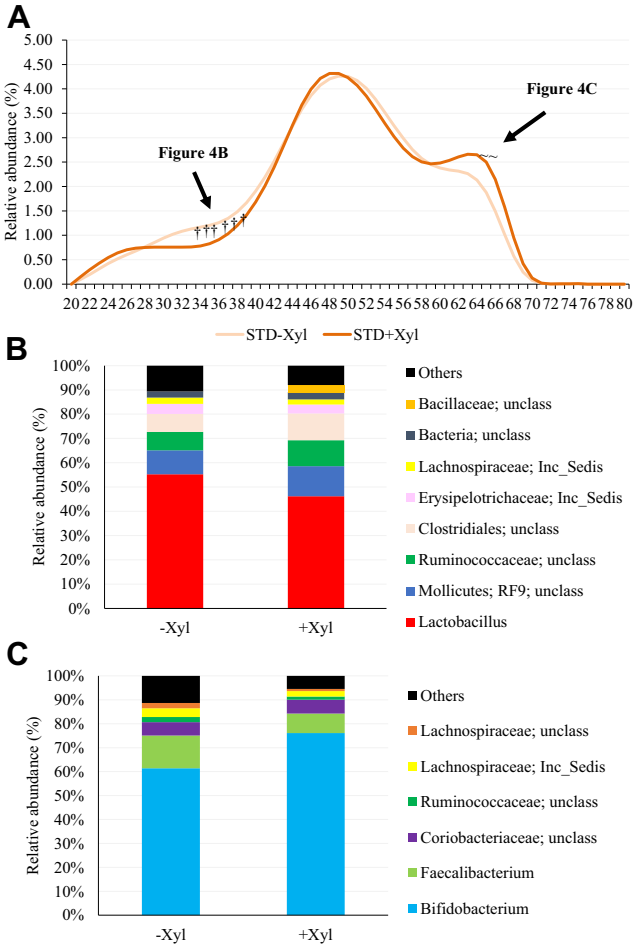


**Figure 3.** (A) Effect of xylanase supplementation in diets with standard dose of phytase (STD) on cecal microbiota of broiler chickens at 28 D of age. Data are means of 5 birds per pen with 8 pens per treatment. The cross marks (†) indicate the % G + C profile which are $P \leq 0.17 > 0.10$, and the tildes (~) indicate the % G + C profile which are $P \leq 0.10 > 0.05$ between the treatments by Student *t*-test. (B) Distribution of the bacterial genera identified in the % G + C fraction 32 to 37%. (C) Distribution of bacterial general identified in the % G + C fraction 64 to 69%.

protein ultimately being fermented in the ceca. However, the concentration of BCFA (considered an indication of protein fermentation) in the ceca of turkeys was lower when compared with broilers suggesting no such problem was apparent in this study. The concentration of all SCFA in the ceca of both poultry species indicate differences in nutrient flow, microbial colonization, and gut maturation (Adebiyi and Olukosi, 2015). At day 28, the proportion of lactic acid to the total SCFA in turkeys was 3-fold greater than for broilers. As the animal ages, flow of rapidly fermentable carbohydrates is reduced and the microbiome biodiversity in the hindgut increases, reducing the production of lactic acid at the expense of other SCFA such as acetic acid, propionic acid, or butyric acid (Oakley et al., 2014; Lee et al., 2017; Wilkinson et al., 2017; Gonzalez-Ortiz et al., 2019). Although all the differences regarding animal species and dietary composition, this may indicate that the microbiome in turkeys at 28 D of age is not as mature compared with broilers.

Phytase concentration or xylanase did not influence the concentrations of any of the SCFA in the cecum of broilers or turkeys. Greater butyric acid concentrations in the digesta of broiler chickens have been reported when xylanase has been supplemented (Masey-O'Neill et al., 2014; González-Ortiz et al., 2016; Lee et al., 2017), but this is not always the case, either for

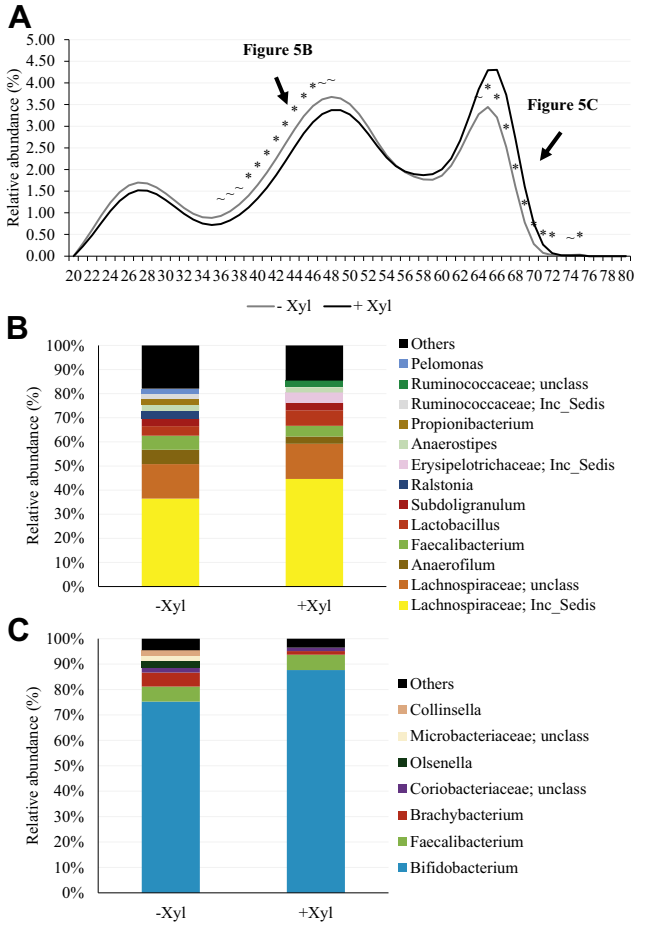


**Figure 4.** (A) Effect of xylanase supplementation combining all doses of phytase on cecal microbiota of turkeys at 28 D of age. Data are means of 5 birds per pen with 8 pens per treatment. The tildes (~) indicate the % G + C profile which are $P \leq 0.10 > 0.05$, and asterisks (*) indicate the % G + C profile which are statistically different ($P \leq 0.05$) between the treatments by Student *t*-test. (B) Distribution of bacterial general identified in the % G + C fraction (44 to 49%) in turkeys fed without xylanase (−Xyl) or with xylanase (+Xyl) combining all doses of phytase. (C) Distribution of bacterial general identified in the % G + C fraction (64–69%) in turkeys fed without xylanase (−Xyl) or with xylanase (+Xyl) combining all doses of phytase.

broilers (Gonzalez-Ortiz et al., 2019) or turkeys (González-Ortiz et al., 2017). However, when the ceca microbiome of control and xylanase fed birds were used as inoculum in an ex vivo fermentation study, the inoculum from xylanase supplemented birds produced significantly more butyric acid than the control birds (Bedford and Apajalahti, 2018), suggesting that the exposure of a broiler to a xylanase augments the capacity of the microbiome to utilize xylan-containing fiber sources, which has implications for intestinal function. Butyric acid is the preferred energy source for colonocytes and influences the integrity of the gut epithelium (Lopetuso et al., 2013).

The effect of phytase on SCFA production is less straight-forward. On the one hand, there is a minimal P requirement for the fermentation of carbohydrates as observed with rumen bacteria (Komisarczuk et al., 1987), thus differences in the concentration of P arriving at the hindgut due to phytase use could be thought to be unimportant on cecal fermentation. However, Ptak et al., (2015) observed that reducing the Ca/P content in the diet was related to a reduction in total SCFA, DL-lactate, and acetic acid in the ileum and that phytase addition increased concentrations of these acids only in birds fed the mineral reduced diets suggesting that phosphate supply may well have been a limiting factor for fermentation in the ileum. Phytase supplementation increased acetic acid and butyric acid concentrations in the cecal digesta of 29-day-old chickens (Smulikowska et al., 2010), whereas phytase supplementation did not affect ileal concentrations of SCFA, but reduced butyric acid concentrations in the excreta of pigs (Metzler-Zebeli et al., 2010). Such variable results suggest the mechanisms by which these effects are brought about are poorly comprehended.

It is worth noting that the concentration of an acid in the ceca does not necessarily reflect the rate of its production by bacteria. Approximately 95 to 99% of SCFA produced in the hindgut are absorbed (von Engelhardt et al., 1998), and the rate of absorption from the intestine into the blood is extremely fast (Pouteau et al., 2008). Moreover, digesta flow is dynamic, and reverse peristalsis may result in variability in local concentrations of the different SCFA (Godwin and Russell, 1997). Thus the poor repeatability and reproducibility of SCFA data is not surprising, and in fact, their value in interpretation of treatment effects is questionable.

Differences in the cecal microbiome between broilers and turkeys were observed. In the current study, the biggest differences were observed in the mid region of the G + C profile (44–49%), where broilers had a lower diversity in bacterial composition compared with turkeys. *Lachnospiraceae (Incertae Sedis)*, unclassified *Lachnospiraceae*, *Lactobacillus*, *Faecalibacterium*, and *Ruminoccocus (Incertae Sedis)* were common to both species, but turkeys had at least 10 other genera vs. the 4 additional genera sequenced in broilers. Bacteria sequenced from the other regions of the G + C profile in broilers and turkeys were similar in identity but not in proportions. Unclassified *Ruminococcaceae*, unclassified *Clostridiales*, *Mollicutes* (RF9 group), and *Bifidobacterium* were the other dominant groups present in both broilers and turkeys. In spite of the variety of techniques and procedures established in different labs, the composition of the microbiome obtained in this study are in agreement with others previously reported. According to Borda-Molina et al., (2018), the most abundant families within the cecum of broilers are *Clostrodicaceae*, *Bacteroidaceae*, *Lactobacillaceae*, and butyrate producers such as *Lachnospiraceae*. Wilkinson et al., (2017) identified *Lactobacillus*, *Streptococcus*, and *Clostridium* XI as the dominant bacteria in the caecum of turkeys.

Xylanase supplementation increased the proportion of *Bifidobacterium* in the ceca of both broilers and turkeys. These results are in agreement with Lee et al., (2017) who also observed an increase of *Bifidobacterium* counts of broiler chickens supplemented with xylanase for 42 D.

Bifidobacteria produce acetate and lactate as end products of sugar fermentation. One of the reasons for such effects may be because of the release of XOS as a result of xylan degradation in the distal sections of the GIT in the presence of xylanase (Bedford, 2018). The beneficial effects of XOS on broiler performance can be explained by the direct stimulation of lactate producing bacteria, such as *Bifidobacterium*. Butyrate producing bacteria can utilize lactate to further ferment to butyrate in the large intestine as hypothesized by De Maesschalck et al., (2015). Although in the current study, the increase in *Bifidobacterium* proportions were not linked to higher concentrations of SCFA measured, other studies have demonstrated that XOS selectively increases the abundance of *Bifidobacterium* and the production of butyrate and acetate (Van Craeyveld et al., 2008). In any case, in this study, xylanase supplementation improved feed conversion ratio in broilers and turkeys (Olukosi et al., 2020), suggesting a possible link between this bacterial group and performance.

In the current study, the highest concentration of phytase coincided with some changes in the microbiota populations in the ceca of broilers. *Faecalibacterium*, unclassified *Clostridiales*, and unclassified *Mollicutes* (RF9 group) were increased with megadosing, whereas the proportion of *Bifidobacterium* decreased. *Faecalibacterium* is one of the most prominent genus in the hindgut and includes butyrate producers. As noted earlier, butyrate provides energy to the colonic mucosa and is known to regulate gene expression, inflammation, differentiation, and apoptosis in host cells (Luo et al., 2013). Regarding *Clostridiales* group, Borda-Molina et al., (2016) identified a genus of *Clostridium* associated with the production of a cysteine phytase. In addition, other bacterial species belonging to *Megasphaera elsdenii* and *Mitsuokella* spp., common members of the rumen microbiota which have the ability to produce phytases (Yanke et al., 1998), were also detected in the ileum and ceca samples from birds receiving diets supplemented with Ca, P, or P with phytase (Borda-Molina et al., 2016), and although these were not identified in the current study, we cannot discard their participation. P, Ca, and phytase influence the microbiome (Witzig et al., 2015; Borda-Molina et al., 2016), and it thus can be speculated that microbiome associated phytase activity complemented phytate degradation by phytase (Palacios et al., 2008). No influence of phytase supplementation on the microbiome in turkeys were observed in the current study, and to the best of our knowledge, no such studies have been reported previously. Broilers possess a greater capacity for InsP6 degradation and hydrolysis for lower inositol phosphates compared with turkeys (Ingelmann et al., 2019; Olukosi et al., 2020), and this difference can be maximized with phytase supplementation. The differences observed between turkeys and broilers in their capacity for InsP6 hydrolysis and P digestibility may be the result of differences in small intestine maturity (Adebiyi and Olukosi, 2015), endogenous P loss, pH along the GIT, passage rate (Rodehutscord and Dieckmann, 2005; Adebiyi and Olukosi, 2015), and differences in their microbiome (Pan and Yu, 2014). Furthermore, when comparing the effects of phytase and xylanase on broilers and turkeys, it is important to recall the differences in diet composition, especially the differences in Ca and P content which has been shown to influence ileal and ceca microbial diversity in broilers (Borda-Molina et al., 2016). It is known that higher doses of Ca in the diets can lead to an increase in pH (Ptak et al., 2015) and lower prececal P digestibility (Adeola and Walk, 2013; Hamdi et al., 2015), which could possibly influence the presence or absence of some bacteria as observed in turkeys by the different G + C profile obtained.

Supplementation of broiler and turkey diets with xylanase resulted in differences in the microbiome in the ceca, which may be due to the release of fermentable XOS, although no effects of xylanase were noted on SCFA in the current study. Supplementation of turkey diets with increasing doses of phytase did not affect the cecal microbiome, contrary to that observed in broilers. There are several factors influencing such differences between bird species; however, the differences in gut maturation, microbiome colonization, feed formulation, passage rate, and pH profile in the different sections of the GIT may have conditioned the microbiome in the cecal of turkeys to the same extent as broilers. For a better understanding of exogenous enzymes effect on broiler and turkey gut function and the microbial community, digesta samples should be studied to determine the rate of oligosaccharide production and InsP6 degradation at different ages until slaughter.

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