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Original Research Article

Broiler gut microbiota and expressions of gut barrier genes affected by cereal type and phytogenic inclusion

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ABSTRACT

The present study assessed the effects of cereal type and the inclusion level of a phytogenic feed additive (PFA) on broiler ileal and cecal gut microbiota composition, volatile fatty acids (VFA) and gene expression of toll like receptors (*TLR*), tight junction proteins, mucin 2 (*MUC2*) and secretory immunoglobulin A (*sIgA*). Depending on cereal type (i.e. maize or wheat) and PFA inclusion level (i.e. 0, 100 and 150 mg/kg diet), 450 one-day-old male broilers were allocated in 6 treatments according to a 2 × 3 factorial arrangement with 5 replicates of 15 broilers each, for 42 d. Significant interactions ($P \leq 0.05$) between cereal type and PFA were shown for cecal digesta *Bacteroides* and *Clostridium* cluster XIVa, ileal digesta propionic and branched VFA, ileal *sIgA* gene expression, as well as cecal digesta branched and other VFA molar ratios. Cereal type affected the cecal microbiota composition. In particular, wheat-fed broilers had higher levels of mucosa-associated *Lactobacillus* ($P_{CT} = 0.007$) and digesta *Bifidobacterium* ($P_{CT} < 0.001$), as well as lower levels of total bacteria ($P_{CT} = 0.004$) and *Clostridia* clusters I, IV and XIVa ($P_{CT} \leq 0.05$), compared with maize-fed ones. In addition, cereal type gave differences in fermentation intensity ($P_{CT} = 0.021$) and in certain individual VFA molar ratios. Wheat-fed broilers had higher ($P \leq 0.05$) ileal zonula occluden 2 (*ZO-2*) and lower ileal and cecal *TLR2* and *sIgA* levels, compared with maize-fed broilers. On the other hand, PFA inclusion at 150 mg/kg had a stimulating effect on microbial fermentation at ileum and a retarding effect in ceca with additional variable VFA molar patterns. In addition, PFA inclusion at 100 mg/kg increased the ileal mucosa expression of claudin 5 (*CLDN5*) ($P_{PFA} = 0.023$) and *MUC2* ($P_{PFA} = 0.001$) genes, and at 150 mg/kg decreased cecal *TLR2* ($P_{PFA} = 0.022$) gene expression compared with the un-supplemented controls. In conclusion, cereal type and PFA affected in combination and independently broiler gut microbiota composition and metabolic activity as well as the expression of critical gut barrier genes including *TLR2*. Further exploitation of these properties in cases of stressor challenges is warranted.

1. Introduction

Consumer preferences for healthy and natural products have resulted in a momentum of rapidly increasing applications of phytogenic components in animal nutrition (Windisch et al., 2008; Brenes and Roura, 2010). In this respect, phytogenic feed additives (PFA) have become increasingly important in broilers due to several positive modulating effects on gut microbiota and metabolic activity (Cao et al., 2010; Cross et al., 2011; Cho et al., 2014; Franciosini et al., 2015; Hashemipour et al., 2016), anti-inflammatory immune response (Hashemipour et al., 2013; Lu et al., 2014; Franciosini et al., 2015; Du et al., 2016) and intestinal barrier properties (Suzuki and Hara, 2011; Zou et al., 2016).

Diet composition is known to be among the key factors affecting PFA efficacy in broilers (Brenes and Roura, 2010; Paraskeuas et al., 2016). Cereals in particular make up the highest percentage of broiler diets. Among the 2 most commonly used cereals worldwide are maize and wheat. Maize is by far the most commonly used

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cereal in broiler diets due to its high nutritional value (Kiarie et al., 2014). Wheat on the other hand, despite the large variability in its physical and chemical properties, is a major energy and protein source in many continents all over the world (Amerah, 2015; Lee et al., 2017). Cereal components such as non-starch polysaccharides are important for their effects on gastrointestinal function and gut ecology (Cao et al., 2010; Svihus et al., 2013; Lee et al., 2017).

In this respect, the aim of this study was to generate further information on the effects of cereal type and dietary PFA administration level and their combinations on gut microbiota composition and metabolic activity as well as on gene expression of gut barrier genes such as toll like receptors (*TLR*), tight junction (TJ) proteins (*ZO-1*, *ZO-2*, *CLDN-1*, *CLDN-2* and *OCLN*), mucin 2 (*MUC2*) and secretory immunoglobulin A (*sIgA*).

2. Materials and methods

2.1. Animals, housing and experimental treatments

For the purpose of the experiment, 450 one-day-old, male Cobb 500 broilers were obtained from a commercial hatchery. Birds were vaccinated at hatch for Marek, Infectious Bronchitis and Newcastle Disease. The experimental protocol was in accordance with the current European Union Directive on the protection of animals used for scientific purposes (EC 43/2007; EU 63/2010) and was approved by the relevant national authority. Birds were euthanized via electrical stunning prior to slaughter. The overall housing and care of the animals conformed to the Faculty of Animal Science and Aquaculture of the Agricultural University of Athens research ethics guidelines.

Chicks were randomly allocated to 6 experimental treatments, described below, for 6 weeks. Each treatment had 5 replicates of 15 broilers each. Each replicate was assigned to a clean floor pen (1 m^2) and birds were raised on rice hulls. The temperature program was set at 32 °C at week 1 and gradually reduced to 23 °C by week 6. Heat was provided with a heating lamp per pen. Except for day 1, a 23-hour-light to 1-hour-dark lighting program was applied during the experiment to ensure maximum access to feed and water.

Depending on the use of maize or wheat as the dietary cereal of basal diets (BD) and the inclusion level of PFA (i.e. 0, 100 and 150 mg/kg diet), the experimental treatments were M0 (maize and no addition of PFA in BD), M100 (maize and PFA added at 100 mg/kg BD), M150 (maize and PFA added at 150 mg/kg BD), W0 (wheat and no addition of PFA in BD); W100 (wheat and PFA added at 100 mg/kg BD) and W150 (wheat and PFA added at 150 mg/kg BD). Diets were in mash form. Diets were formulated so as to meet boiler requirements for starter (1 − 14 d), grower (15 − 28 d) and finisher (29 − 42 d) growth periods by taking into account Cobb 500 recommendations for Europe. The PFA (Digestarom Poultry, Biomin Phytogenics GmbH, Germany) contained different modules (components), based on herbs, spices and essential oils characterised by menthol and anethole. The PFA had an active ingredient concentration of 350 g/kg. On a weekly basis, PFA was incorporated in the BD at the expense of maize or wheat. Throughout the experiment, experimental diets and water were available *ad libitum*.

2.2. Tissue sampling for subsequent analyses

At 42 d of age, 10 broilers per treatment (i.e. 2 birds per replicate cage) were randomly selected and ileum and ceca samples were carefully excised aseptically, snap frozen in liquid nitrogen and subsequently stored at −80 °C. From these samples half (i.e. 5 birds per treatment) were used for mucosa and digesta DNA isolation and volatile fatty acids (VFA) analysis and the other half were used for mucosa RNA isolation.

2.3. DNA isolation and quantification of luminal and mucosa associated ileal and cecal microbiota

From the deep-frozen ileum and ceca collected previously from 5 birds per treatment (i.e. one bird per replicate cage), the ileal and cecal luminal digesta were aseptically removed with tweezers after a longitudinal opening performed with a sterile scalpel, collected and placed in sterile falcon tubes and immediately frozen in liquid nitrogen and subsequently stored at −80 °C. Following the removal of ileal and cecal luminal digesta, the intestinal segments were initially washed 2 times via subsequent immersions and mild hand shaking in 25 mL ice-cold sterile phosphate buffer saline (PBS). Afterwards, each intestinal segment was washed 3 times consecutively with 15 mL ice-cold sterile saline containing 0.1% (wt/wt) Tween 80 in 50 mL conical tubes by vigorously shaking 1 min per wash. The three 15 mL washes were pooled and centrifuged at 10,000 × *g* at 4 °C for 30 min. The resulting mucosa-associated cell pellet was removed and placed in a sterile Eppendorf tube that was then frozen in liquid nitrogen and stored at −80 °C.

Total DNA was isolated from the ileal and cecal luminal digesta as well as from mucosa-associated cell pellet using a suitable commercial kit (PSP Spin Stool DNA Kit, Stratec Molecular GmbH, Berlin, Germany). The lysis protocol was optimized by incorporating an additional lysozyme (50 mg/mL) digestion step at 37 °C for 30 min. For each sample, DNA was eluted in 200 μL elution buffer and the quality and quantity of the preparations were determined by spectrophotometry (NanoDrop-1000, Thermo Fisher Scientific, Waltham, UK) and stored at −30 °C.

DNA samples were analyzed for the following microbiota constituents: total bacteria, *Escherichia coli*, *Lactobacillus* spp., *Bifidobacterium* spp., *Bacteroides* spp., *Clostridium* cluster I, *Clostridium* cluster IV and *Clostridium* cluster XIVa. Suitable primers targeting the 16S rRNA gene for each one of the target microbiota constituents were selected from the relevant scientific literature (Table 1). Primer specificity was confirmed using BLAST and were obtained from IDT (Integrated DNA Technologies Inc, IA, USA).

Reference microbial strains following appropriate culture and subsequent DNA isolation were used for primer verification and standard curve construction (Table 1). Standard curves were constructed from 10-fold serial dilutions of known concentrations of genomic DNA from each reference strain and plotted against the respective threshold cycle value. Subsequently, sample microbial target DNA quantity was determined and expressed as $\log_{10}$ cells per gram of digesta content or mucosa associated cell pellet by calculating the number of cells from the quantity of DNA divided with the mean mass of the corresponding microbial genome size listed in the National Center for Biotechnology Information (NCBI).

Real-time PCR was performed using an ABI 7500 Real-time PCR system (Applied Biosystems, CA) using optical grade 96-well plates (PEQLAB Biotechnologie GmbH, Erlangen, Germany). All reactions were made at a 20 μL final volume and consisted of 10 μL of 2 × Green Dye master mix (Rovalab GmbH, Teltow, Germany), forward and reverse primers each at final concentration of 200 or 300 nmol/L (i.e., 0.4 or 0.6 μL of a 10 μmol/L stock), 1 μL of bovine serum albumin (20 μg/mL), 2 μL of template DNA (50 ng sample DNA/reaction), 0.2 μL passive ROX reference dye (5 μmol/L) at 50 nmol/L final concentration, and PCR grade water up to 20 μL final reaction volume. The amplification program used was one cycle of 95 °C for 10 min, 40 cycles of 95 °C for 30 s, primer specific annealing temperature for 60 s, then 72 °C for 33 s. Following amplification, a melt curve analysis was constructed to analyze the melting profile of the amplified product.

Table 1
Primers targeting 16S rRNA gene used for the determination of ileal and cecal mucosa-associated and luminal digesta microbiota composition, by quantitative real-time PCR.

Target	Primer sequence (5′–3′)	Annealing temperature, °C	Reference
Total bacteria	F: ACTCCTACGGGAGGCAGCAG R: ATTACCGCGGCTGCTGG	60	Clifford et al. (2012)
Escherichia coli	F: CATGCCGCGTGTATGAAGAA R: GGGTAACGTCAATGAGCAAAGG	60	Silkie and Nelson, 2009
Lactobacillus spp.	F: GAGGCAGCAGTAGGGAATCTTC R: GGCCAGTTACTACCTCTATCCTTCTTC	60	Delroisse et al. (2008)
Bifidobacterium spp.	F: CGCGTCYGGTGTGAAAG R: CCCCACATCCAGCATCCA	58	Peinado et al. (2013)
Bacteroides spp.	F: GAGAGGAAGGTCCCCCAC R: CGCTACTTGGCTGGTTCAG	58	Peinado et al., 2013
Clostridium cluster I	F: TACCHRAGGAGGAAGCCAC R: GTTCTTCCTAATCTCTACGCAT	56	Boroojeni et al., 2014
C. cluster IV	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA	52	Matsuki et al. (2004)
C. cluster XIVa	F: ACTCCTACGGGAGGCAGC R: CTTCTTAGTCAGGTACCGTCAT	60	Schwiertz et al. (2010)

2.4. Volatile fatty acid concentration

Ileum and cecal digesta VFA concentrations were determined in duplicate in the supernatants of ileal and cecal digesta homogenates after centrifugation at 12,000 × *g* for 10 min at 4 °C. Concentrations of acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic, isocaproic, and heptanoic acids were determined by capillary gas chromatography using a Perkin–Elmer Autosystem XL gas chromatograph equipped with a 30 m × 0.25 mm inside diameter Nukol column (Supelco, Sigma–Aldrich, St. Louis, MO) and a flame ionization detector as described by Mountzouris et al. (2007).

2.5. RNA isolation and determination of relative gene expressions in ileal and cecal mucosa

The middle section (15 cm) of ileum and the whole ceca were longitudinally opened and the luminal digesta was removed. Subsequently, digesta-free sections were washed two times consecutively in 25 mL ice-cold PBS-EDTA (pH = 7.2) and each mucosal layer was scraped off with a micro slide and placed in sterile Eppendorf tube. Afterwards, Trifast Reagent (PEQLAB Biotechnologie GmbH, Erlangen, Germany) was used to extract RNA from the ileal and cecal mucosa, according to the manufacturer's protocol. RNA quantity was determined by spectrophotometry (NanoDrop-1000, Thermo Fisher Scientific, Waltham, United Kingdom). RNA integrity was assessed by agarose gel electrophoresis.

Prior to complementary DNA (cDNA) synthesis, DNase treatment was applied. Ten μg of RNA were treated with 1 U of DNase I (M0303, New England Biolabs Inc, Ipswich, UK) and 10 μL of 10 × DNase buffer for 1 h at 37 °C. The DNase was inactivated by the addition of 1 μL of 0.5 mol/L EDTA at 75 °C for 10 min. RNA integrity was assessed by agarose gel electrophoresis. For cDNA preparation, 500 ng of total RNA from each sample were reverse transcribed to cDNA by PrimeScript RT Reagent Kit (Perfect Real Time, Takara Bio Inc., Shiga-Ken, Japan) according to the manufacturer's recommendations. All cDNAs were then stored at −20 °C.

Respective cDNA samples were assayed for expressions of the following *Gallus gallus* genes: *TLR* (*TLR2, TLR4*), claudins (*CLDN1* and *CLDN5*), occludin (*OCLN*), cytosolic proteins zonula occludens (*ZO1* and *ZO2*), *MUC2*, *sIgA*, nuclear factor kappaB (*NF-κB*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Suitable primers were designed using the GenBank sequences deposited on the NCBI shown in Table 2. Primers were checked using the PRIMER BLAST algorithm against *Gallus gallus* mRNA databases to ensure that there was a unique amplicon.

Real-time PCR was performed in 96 well microplates with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and a KAPA SYBR FAST qPCR Kit (KAPA Biosystems, Wilmington, MA, USA). Each reaction contained 12.5 ng RNA equivalents as well as 200–250 nmol/L of forward and reverse primers for each gene. The reactions were incubated at 95 °C for 3 min, followed by 40 cycles at 95 °C for 5 s 60 or 62 °C (depends on the target gene) for 20 s, 72 °C for 33 s. This was followed by a melt curve analysis to determine the reaction specificity. Each sample was measured in duplicates. Relative expression ratios of target genes were calculated according to Pfaffl, (2001) using *GAPDH* as a reference gene.

2.6. Statistical analysis

Experimental data were tested for normality using the Kolmogorov–Smirnov test and found to be normally distributed. Data were analyzed with the general linear model (GLM) – general factorial ANOVA procedure using cereal type (maize and wheat) and PFA inclusion level (i.e. 0, 100 and 150 mg/kg diet) as fixed factors. Statistically significant effects were further analyzed and means were compared using Tukey's honestly significant difference multiple comparison procedure. Statistical significance was determined at $P \leq 0.05$. All statistical analyses were done using the SPSS for Windows Statistical Package Program (SPSS Inc., Chicago, IL).

3. Results

3.1. Ileal and cecal microbiota composition

The mucosa-associated levels of bacteria examined at the ileum and ceca were not affected ($P > 0.05$) by cereal type and PFA inclusion level (Tables 3 and 4), except in the case of cecal mucosa-associated *Lactobacillus* spp. that was significantly ($P_{CT} = 0.007$) higher in broilers fed wheat based diets compared with maize-fed ones (Table 4).

Ileal digesta total bacteria concentration as well as *Lactobacillus* spp. *E. coli*, *Bacteroides* spp., and *Clostridium* cluster XIVa levels were not affected ($P > 0.05$) by cereal type or PFA supplementation level (Table 5). Significant interactions between cereal type and PFA administration level were shown for cecal digesta *Bacteroides* spp. ($P_{CT \times PFA} = 0.025$) and *Clostridium* cluster IV ($P_{CT \times PFA} = 0.048$). In addition, cecal digesta total bacteria ($P_{CT} = 0.004$), as well as *Clostridium* cluster I ($P_{CT} = 0.019$), *Clostridium* cluster IV ($P_{CT} \leq 0.001$)

Table 2
Oligonucleotide primers used for the study of gene expression of selected targets by quantitative real time PCR.

Target	Primer sequence (5′–3′)	Annealing temperature, °C	PCR product size, bp	GenBank accession No.
GAPDH	F:GCTGAATGGGAAGCTTACTG R: AAGGTGGAGGAATGGCTG	60	216	NM_204305.1
ZO-1	F:TAAAGCCATTCCTGTAAGCC R: GTTTCACCTTTCTCTTTGTCC	60	243	XM_015278981.1
ZO-2	F: GGCAAATCATTGAGCAGGA R: ATTGATGGTGGCTGTAAAGAG	60	239	XM_015280247.1
CLDN1	F: CTGATTGCTTCCAACCAG R: CAGGTCAAACAGAGGTACAAG	59	140	NM_001013611.2
CLDN5	F: CATCACTTCTCCTTCGTCAGC R: GCACAAAGATCTCCCAGGTC	59	111	NM_204201.1
OCLN	F: TCATCGCCTCCATCGTCTAC R: TCTTACTGCGCGTCTTCTGG	62	240	NM_205128.1
TLR2	F: CTTGGAGATCAGAGTTTGGA R: ATTTGGGAATTTGAGTGCTG	62	238	XM_015301380.1
TLR4	F: GTCTCTCCTTCCTTACCTGCTGTTC R: AGGAGGAGAAAGACAGGGTAGGTG	65	187	NM_001030693.1
NF-κB	F: TGTGGTTGTCAGGATGGTC R: GGTCTGGTAAAGGTCATTTCTC	62	273	XM_015285418.1
MUC2	F: GCTGATTGTCACTCACGCCTT R: ATCTGCCTGAATCACAGGTGC	62	442	XM_421035
sIgA	F: GTCACCGTCACCTGGACTACA R: ACCGATGGTCTCCTTCACATC	59	192	S40610

GAPDH = glyceraldehyde 3-phosphate dehydrogenase; *ZO-1, -2* = zona occludens 1, 2; *CLDN1, 5* = claudins 1, 5; *OCLN* = occluding; *NF-κB* = nuclear factor kappa light-chain-enhancer of activated B cells; *TLR2, 4* = Toll-like receptors 2, 4; *MUC2* = mucin 2; *sIgA* = secretory immunoglobulin A.
F: forward, R: reverse.

and *Clostridium* cluster XIVa ($P_{CT} = 0.003$) levels were significantly lower in broilers fed wheat-based diets compared with those fed maize-fed ones. However, cecal digesta levels of *Bifidobacterium* spp. ($P_{CT} \leq 0.001$) were significantly higher in broilers fed wheat compared with maize-based diets (Table 6).

3.2. Volatile fatty acids

Significant interactions were shown between cereal type and PFA inclusion level for propionic acid ($P_{CT \times PFA} = 0.016$) and branched VFA ($P_{CT \times PFA} = 0.030$) molar ratios (Table 7). The type of cereal did not affect ileal digesta VFA concentration and molar ratios. However, PFA inclusion level affected the ileal digesta total VFA concentration ($P_{PFA} \leq 0.001$) and the broilers on the high PFA level (i.e. 150 mg/kg diet) had higher concentration compared with the un-supplemented control and the 100 mg/kg dietary PFA level. Moreover, PFA supplementation level affected the molar ratios of propionic acid ($P_{PFA} = 0.013$) and branched VFA ($P_{PFA} = 0.034$) and broilers on 100 mg PFA/kg diet level had higher values compared with the 150 mg PFA/kg diet level and the un-supplemented

Table 3
Ileal mucosa-associated bacteria levels of 42-day-old broilers.

Ileal mucosa-associated bacteria (log_{10} cells/g mucosa-associated cell pellet)[1]	Total bacteria	*Lactobacillus* spp.	*Clostridium* cluster XIVa
Type of cereal[2]			
Maize (M)	7.28	6.75	5.66
Wheat (W)	7.40	6.40	5.76
PFA supplementation, mg/kg diet[3]			
0	7.52	6.62	5.82
100	7.23	6.33	5.53
150	7.26	6.77	5.78
Interaction (treatments)[4]			
M0	7.37	6.74	5.74
M100	7.34	6.74	5.59
M150	7.12	6.76	5.64
W0	7.67	6.49	5.90
W100	7.13	5.93	5.46
W150	7.40	6.78	5.92
SEM	0.170	0.254	0.200
P-values			
P_{CT}	0.384	0.109	0.527
P_{PFA}	0.195	0.236	0.298
$P_{CT \times PFA}$	0.245	0.264	0.576

M0 = maize-soy bean meal (SBM) basal diet with no other additions; M100 = maize-SBM basal diet with addition of 100 mg PFA/kg diet; M150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; W0 = wheat-SBM basal diet with no other additions; W100 = wheat-SBM basal diet with addition of 100 mg PFA/kg diet; W150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; SEM = pooled standard error of means; CT = cereal type; PFA = phytogenic feed additive.
[1] All microbial cfu data were transformed to respective log_{10} values before being analyzed.
[2] Basal diets based on maize (M) or wheat (W). Data shown per CT represent treatment means from 15 broilers (e.g. 5 from treatment M0 + 5 from treatment M100 + 5 from treatment M150).
[3] Phytogenic supplementation (0, 100 and 150 mg/kg diet). Data shown for PFA represent means from 10 replicate pens (e.g. 5 from treatment M0 + 5 from treatment W0).
[4] Interaction means (treatments) for 5 battery cages per treatment.

Table 4
Cecal mucosa-associated bacteria levels of 42-day-old broilers.

Cecal mucosa-associated bacteria (log_{10} cells/g mucosa-associated cell pellet)[1]	Total bacteria	*Lactobacillus* spp.	*Bacteroides* spp.	*Clostridium* cluster IV	*Clostridium* cluster XIVa
Type of cereal[2]					
Maize (M)	8.68	5.97[B]	7.93	8.23	8.19
Wheat (W)	8.62	6.42[A]	8.16	8.16	8.25
PFA supplementation, mg/kg diet[3]					
0	8.57	6.05	8.11	8.26	8.21
100	8.58	6.09	7.90	8.12	8.19
150	8.78	6.44	8.13	8.22	8.27
Interaction (treatments)[4]					
M0	8.53	5.83	7.95	8.32	8.15
M100	8.68	5.83	7.93	8.14	8.15
M150	8.81	6.26	7.91	8.25	8.27
W0	8.61	6.28	8.26	8.20	8.28
W100	8.49	6.35	7.87	8.10	8.22
W150	8.76	6.62	8.35	8.20	8.27
SEM	0.135	0.184	0.181	0.132	0.135
P-values					
P_{CT}	0.592	0.007	0.141	0.526	0.414
P_{PFA}	0.233	0.089	0.390	0.563	0.699
$P_{CT \times PFA}$	0.610	0.901	0.368	0.953	0.814

M0 = maize-soy bean meal (SBM) basal diet with no other additions; M100 = maize-SBM basal diet with addition of 100 mg PFA/kg diet; M150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; W0 = wheat-SBM basal diet with no other additions; W100 = wheat-SBM basal diet with addition of 100 mg PFA/kg diet; W150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; SEM = pooled standard error of means; CT = cereal type; PFA = phytogenic feed additive.
[A, B] Within a column, means with different superscripts differ at $P < 0.01$.
[1] All microbial cfu data were transformed to respective log_{10} values before being analyzed.
[2] Basal diets based on maize (M) or wheat (W). Data shown per CT represent treatment means from 15 broilers (e.g. 5 from treatment M0 + 5 from treatment M100 + 5 from treatment M150).
[3] Phytogenic supplementation (0, 100 and 150 mg/kg diet). Data shown for PFA represent means from 10 replicate pens (e.g. 5 from treatment M0 + 5 from treatment W0).
[4] Interaction means (treatments) for 5 battery cages per treatment.

control. Finally, PFA level 150 mg/kg diet resulted in lower molar ratio of other-VFA compared with the un-supplemented controls and the dietary supplementations of 100 mg PFA/kg diet.

A significant interaction between diet type and PFA administration was shown for branched VFA ($P_{CT \times PFA} = 0.007$) molar ratio. Cereal type significantly affected total VFA concentration ($P_{CT} = 0.021$) as well as the butyric acid molar ratio ($P_{CT} = 0.012$) both of which were higher in wheat-based diets compared with maize based ones (Table 8). On the other hand, cereal type significantly affected the molar ratios of acetic acid ($P_{CT} = 0.040$), branched VFA ($P_{CT} \leq 0.001$) and other VFA ($P_{CT} = 0.001$) with the lower ratios seen in wheat-based diets compared with maize

Table 5
Ileal digesta microbiota composition of 42-day-old broilers.

Ileal digesta content (log_{10} cells/g digesta)[1]	Total bacteria	*Escherichia coli*	*Lactobacillus* spp.	*Bacteroides* spp.	*Clostridia* cluster XIVa
Type of cereal[2]					
Maize (M)	7.96	5.03	6.53	4.26	7.46
Wheat (W)	7.78	5.23	6.33	4.14	7.32
PFA supplementation, mg/kg diet[3]					
0	8.01	5.28	6.51	4.15	7.49
100	7.83	5.07	6.38	3.92	7.38
150	7.77	5.05	6.40	4.53	7.29
Interaction (treatments)[4]					
M0	8.10	5.53	6.56	4.49	7.55
M100	7.95	4.79	6.47	3.75	7.51
M150	7.83	4.77	6.58	4.54	7.31
W0	7.92	5.03	6.47	3.81	7.43
W100	7.71	5.35	6.28	4.09	7.24
W150	7.70	5.32	6.23	4.52	7.27
SEM	0.193	0.396	0.233	0.247	0.231
P-values					
P_{CT}	0.263	0.542	0.284	0.563	0.456
P_{PFA}	0.440	0.812	0.828	0.063	0.695
$P_{CT \times PFA}$	0.960	0.322	0.858	0.133	0.886

M0 = maize-soy bean meal (SBM) basal diet with no other additions; M100 = maize-SBM basal diet with addition of 100 mg PFA/kg diet; M150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; W0 = wheat-SBM basal diet with no other additions; W100 = wheat-SBM basal diet with addition of 100 mg PFA/kg diet; W150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; SEM = pooled standard error of means; CT = cereal type; PFA = phytogenic feed additive.
[1] All microbial cfu data were transformed to respective log_{10} values before being analyzed.
[2] Basal diets based on maize (M) or wheat (W). Data shown per CT represent treatment means from 15 broilers (e.g. 5 from treatment M0 + 5 from treatment M100 + 5 from treatment M150).
[3] Phytogenic supplementation (0, 100 and 150 mg/kg diet). Data shown for PFA represent means from 10 replicate pens (e.g. 5 from treatment M0 + 5 from treatment W0).
[4] Interaction means (treatments) for 5 battery cages per treatment.

Table 6
Cecal digesta microbiota composition of 42-day-old broilers.

Cecal digesta content (log_{10} cells/g digesta)[1]	Total bacteria	*Escherichia coli*	*Lactobacillus* spp.	*Bifidobacterium* spp.	*Bacteroides* spp.	*Clostridium* cluster I	*Clostridium* cluster IV	*Clostridium* cluster XIVa
Type of cereal[2]								
Maize (M)	10.09^{A}	8.16	7.40	5.26^{B}	8.01	7.89^{a}	9.25^{A}	9.74^{A}
Wheat (W)	9.85^{B}	8.42	7.65	6.73^{A}	8.04	7.62^{b}	8.73^{B}	9.56^{B}
PFA supplementation, mg/kg diet[3]								
0	9.86	8.21	7.45	6.08	7.85	7.73	8.90	9.56
100	10.04	8.29	7.48	5.86	8.14	7.66	9.03	9.71
150	10.01	8.37	7.66	6.06	8.08	7.86	9.04	9.68
Interaction (treatments)[4]								
M0	10.01	8.04	7.49	5.48	7.91^{bc}	7.99	9.36^{a}	9.72
M100	10.22	8.08	7.27	5.18	8.25^{ab}	7.86	9.31^{a}	9.79
M150	10.03	8.37	7.46	5.13	7.86^{c}	7.81	9.09^{ab}	9.70
W0	9.70	8.39	7.41	6.68	7.79^{c}	7.48	8.44^{c}	9.40
W100	9.85	8.49	7.69	6.53	8.03^{abc}	7.46	8.76^{bc}	9.63
W150	10.00	8.38	7.86	6.99	8.31^{a}	7.91	9.00^{ab}	9.65
SEM	0.089	0.219	0.182	0.193	0.123	0.132	0.157	0.064
P-values								
P_{CT}	0.004	0.163	0.109	<0.001	0.719	0.019	<0.001	0.003
P_{PFA}	0.110	0.766	0.484	0.455	0.063	0.344	0.593	0.067
$P_{CT \times PFA}$	0.132	0.628	0.327	0.220	0.025	0.069	0.048	0.122

M0 = maize-soy bean meal (SBM) basal diet with no other additions; M100 = maize-SBM basal diet with addition of 100 mg PFA/kg diet; M150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; W0 = wheat-SBM basal diet with no other additions; W100 = wheat-SBM basal diet with addition of 100 mg PFA/kg diet; W150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; SEM = pooled standard error of means; CT = cereal type; PFA = phytogenic feed additive.
[a, b] Within a column, means with different superscripts differ at $P < 0.05$.
[A, B, C] Within a column, means with different superscripts differ at $P < 0.01$.
[1] All microbial cfu data were transformed to respective log_{10} values before being analyzed.
[2] Basal diets based on maize (M) or wheat (W). Data shown per CT represent treatment means from 15 broilers (e.g. 5 from treatment M0 + 5 from treatment M100 + 5 from treatment M150).
[3] Phytogenic supplementation (0, 100 and 150 mg/kg diet). Data shown for PFA represent means from 10 replicate pens (e.g. 5 from treatment M0 + 5 from treatment W0).
[4] Interaction means (treatments) for 5 battery cages per treatment.

ones. The PFA inclusion level significantly affected total VFA concentration ($P_{PFA} = 0.041$) and the butyric acid molar ratio ($P_{PFA} = 0.029$) and broilers on the 150 mg PFA/kg diet level had lower concentration compared with the un-supplemented controls (Table 8).

3.3. Tight junction proteins, toll like receptor(s), nuclear factor kappa B, mucin 2 and secretory immunoglobulin A expression levels

Gene expressions of *ZO-1*, *CLDN1*, *OCLN*, *TLR4*, *NF-κB* and *MUC2* in ileal mucosa were not affected ($P > 0.05$) by cereal type and PFA

Table 7
Volatile fatty acids (VFA) in ileum content of 42-day-old broilers (mmol/kg wet digesta).

Item	Ileal content VFA[1]					
	Total VFA	Acetic, %	Propionic, %	Butyric, %	Branched VFA, %	Other VFA, %
Type of cereal[2]						
Maize (M)	7.45	64.11	6.27	21.34	2.31	5.96
Wheat (W)	8.48	67.31	5.00	18.92	3.16	5.60
PFA supplementation, mg/kg diet[3]						
0	6.83^{B}	66.82	5.14^{ab}	19.94	1.67^{b}	6.42^{a}
100	6.09^{B}	62.07	7.61^{a}	20.34	3.60^{a}	6.38^{a}
150	10.97^{A}	68.24	4.15^{b}	20.11	2.95^{ab}	4.54^{b}
Interaction (treatments)[4]						
M0	7.22	66.77	4.59^{b}	21.27	1.43^{b}	5.93
M100	5.81	59.20	10.22^{a}	21.84	2.10^{b}	6.65
M150	9.32	66.39	4.00^{b}	20.92	3.42^{b}	5.28
W0	6.45	66.88	5.69^{b}	18.61	1.91^{b}	6.91
W100	6.37	64.95	5.01^{b}	18.84	5.10^{a}	6.11
W150	12.61	66.77	4.31^{b}	19.31	2.48^{b}	3.80
SEM	1.071	3.384	1.097	3.389	0.698	0.811
P-values						
P_{CT}	0.253	0.260	0.171	0.390	0.149	0.600
P_{PFA}	<0.001	0.183	0.013	0.993	0.034	0.046
$P_{CT \times PFA}$	0.176	0.704	0.016	0.978	0.030	0.329

M0 = maize-soy bean meal (SBM) basal diet with no other additions; M100 = maize-SBM basal diet with addition of 100 mg PFA/kg diet; M150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; W0 = wheat-SBM basal diet with no other additions; W100 = wheat-SBM basal diet with addition of 100 mg PFA/kg diet; W150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; SEM = pooled standard error of means; CT = cereal type; PFA = phytogenic feed additive.
[a, b] Within a column, means with different superscripts differ at $P < 0.05$.
[A, B] Within a column, means with different superscripts differ at $P < 0.01$.
[1] Total VFA: acetic + propionic + butyric + branched VFA + other VFA; Branched VFA: isobutyric + isovaleric + isocaproic; Other VFA: valeric + caproic + heptanoic.
[2] Basal diets based on maize (M) or wheat (W). Data shown per CT represent treatment means from 15 broilers (e.g. 5 from treatment M0 + 5 from treatment M100 + 5 from treatment M150).
[3] Phytogenic supplementation (0, 100 and 150 mg/kg diet). Data shown for PFA represent means from 10 replicate pens (e.g. 5 from treatment M0 + 5 from treatment W0).
[4] Interaction means (treatments) for 5 battery cages per treatment.

Table 8
Volatile fatty acids (VFA) in cecal content of 42-day-old broilers (mmol/kg wet digesta).

Item	Cecal digesta VFA[1]					
	Total VFA	Acetic, %	Propionic, %	Butyric, %	Branched VFA, %	Other VFA, %
Type of cereal[2]						
Maize (M)	92.00^{b}	63.36^{a}	6.49	25.97^{b}	2.17^{B}	2.00^{B}
Wheat (W)	111.43^{a}	59.51^{b}	6.29	31.46^{a}	1.19^{A}	1.55^{A}
PFA supplementation, mg/kg diet[3]						
0	115.06^{a}	59.60	5.64	31.72^{a}	1.37	1.67
100	101.11^{ab}	60.73	6.49	29.64^{ab}	1.43	1.72
150	88.98^{b}	63.98	7.04	24.79^{b}	2.24	1.94
Interaction (treatments)[4]						
M0	114.35	62.94	5.31	28.51	1.45^{B}	1.78
M100	91.04	62.91	7.04	26.56	1.64^{B}	1.84
M150	70.63	64.22	7.11	22.85	3.42^{A}	2.40
W0	115.78	56.26	5.97	34.92	1.30^{B}	1.56
W100	111.18	58.54	5.94	32.72	1.21^{B}	1.60
W150	107.32	63.74	6.97	26.73	1.06^{B}	1.49
SEM	9.646	2.171	0.592	2.472	0.351	0.152
P-values						
P_{CT}	0.021	0.040	0.686	0.012	0.000	0.001
P_{PFA}	0.041	0.133	0.078	0.029	0.094	0.174
$P_{CT \times PFA}$	0.209	0.368	0.345	0.854	0.007	0.056

M0 = maize-soy bean meal (SBM) basal diet with no other additions; M100 = maize-SBM basal diet with addition of 100 mg PFA/kg diet; M150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; W0 = wheat-SBM basal diet with no other additions; W100 = wheat-SBM basal diet with addition of 100 mg PFA/kg diet; W150 = maize-SBM basal diet with addition of 150 mg PFA/kg diet; SEM = pooled standard error of means; CT = cereal type; PFA = phytogenic feed additive.
[a, b] Within a column, means with different superscripts differ at $P < 0.05$.
[A, B] Within a column, means with different superscripts differ at $P < 0.01$.
[1] Total VFA: acetic + propionic + butyric + branched VFA + other VFA; Branched VFA: isobutyric + isovaleric + isocaproic; Other VFA: valeric + caproic + heptanoic.
[2] Basal diets based on maize (M) or wheat (W). Data shown per CT represent treatment means from 15 broilers (e.g. 5 from treatment M0 + 5 from treatment M100 + 5 from treatment M150).
[3] Phytogenic supplementation (0, 100 and 150 mg/kg diet). Data shown for PFA represent means from 10 replicate pens (e.g. 5 from treatment M0 + 5 from treatment W0).
[4] Interaction means (treatments) for 5 battery cages per treatment.

addition (Table 9). However, a significant interaction between cereal type and PFA administration ($P_{CT \times PFA} = 0.021$) was shown for *sIgA*. In particular, higher expression levels of sIgA were shown for broilers of treatment M100 (2.01) compared to broilers of treatments M0 (0.75), W100 (0.74) and W150 (0.68). Treatments M150 (1.78) and W (0.96) were intermediate and not different from the treatments above. Cereal type significantly affected *ZO-2* ($P_{CT} = 0.014$), and broilers fed wheat-based diets had higher expression compared with maize-fed ones. Moreover, broilers fed wheat-based diets had lower expression levels of *TLR2* ($P_{CT} = 0.004$) and *sIgA* ($P_{CT} = 0.003$) compared with those fed maize-based diets. The PFA administration level significantly affected ileal mucosa expression levels of *CLDN5* ($P_{PFA} = 0.023$) and *MUC2* ($P_{PFA} = 0.001$) and broilers fed supplemented diet at 100 mg PFA/kg had higher expression compared with the un-supplemented control (Table 9).

In cecal mucosa the gene expression levels of *ZO-1*, *CLDN5*, *OCLN*, *TLR4*, *NF-κB* and *MUC2* were not affected ($P > 0.05$) by cereal type and PFA inclusion level (Table 9). However, cereal type affected *CLDN1* ($P_{CT} = 0.035$), *TLR2* ($P_{CT} = 0.001$) and *sIgA* ($P_{CT} = 0.002$) and broilers fed wheat-based diets showed lower expression levels compared with maize-fed ones. The PFA inclusion level significantly affected cecal mucosa expression levels of *TLR2* ($P_{PFA} = 0.022$), and broilers supplemented PFA at 150 mg/kg diet had lower levels compared with the un-supplemented controls and dietary supplementation of 100 mg PFA/kg diet (Table 9).

4. Discussion

Current research highlights the role of diet as one of the most important factors affecting overall gut function and health (Brenes and Roura, 2010; Celi et al., 2017; Ducatelle et al., 2018). In particular, dietary bioactive constituents are purported to act directly or indirectly on continuously interacting elements that define gut ecology such as gut microbiota composition and metabolic activity, gut integrity and inflammatory status (Choct, 2009; Suzuki and Hara, 2011; Lee et al., 2017). This work aimed to progress further previous findings on broiler performance, nutrient digestibility, blood and meat total antioxidant capacity (Paraskeuas et al., 2016, 2017) and focus on the effects of cereal type and PFA supplementation level on broiler gut microbiota and expressions of critical gut barrier genes.

In this work, mucosa-associated and gut lumen content predominant gut microbiota members of the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*, known to account for more than 90% of the gut microbiota in poultry (Lu et al., 2003; Lan et al., 2004) were analyzed by qPCR. From the gut microbiota members examined, it was shown that the cereal type used to formulate the diets interacted with PFA inclusion level and impacted cecal digesta levels of *Bacteroides* spp. and *Clostridium* cluster IV. In particular, depending on the cereal used the higher *Bacteroides* spp. levels were shown at different PFA inclusion level (i.e. M100 vs. W150). In addition, the *Clostridium* cluster IV levels were more responsive to increase with PFA inclusion level in broilers fed wheat compared with maize-based diets. It is known that wheat composition differs from maize with regards to certain carbohydrate components (e.g. non-starch polysaccharides such as arabinoxylans and beta-glucans). In turn, these components may affect gut microbiota composition and metabolic activities (Apajalahti et al., 2004; Lee et al., 2017).

In this study, it was shown that irrespective of PFA inclusion, wheat impacted the cecal digesta microbiota by reducing total bacteria concentration as well as *Clostridia* clusters I, IV and XIVa compared with maize. In addition, wheat apart from increasing the cecal mucosa associated *Lactobacillus* levels, also displayed a strong bifidogenic potential in the digesta, compared with maize. Wheat effects on members of broiler gut microbiota have been reported for various microbiota members such as *Clostridium, Lactobacillus*

Table 9
Relative gene expression of tight junction proteins, toll like receptor(s), nuclear factor kappaB, mucin 2 and secretory immunoglobulin alpha in ileal and cecal mucosa of 42-day-old broilers.

Item	Gene[1]	Type of cereal (CT)[2]		PFA supplementation, mg/kg diet[3]			SEM	*P*-values		
		M	W	0	100	150		CT	PFA	CT × PFA
Ileal										
	ZO-1	1.00	1.15	1.01	1.24	0.97	0.227	0.435	0.448	0.052
	ZO-2	0.85^{b}	1.23^{a}	0.95	1.18	1.01	0.181	0.014	0.424	0.519
	CLDN1	1.13	1.32	1.06	1.62	0.99	0.438	0.608	0.310	0.818
	CLDN5	1.10	1.10	0.72^{b}	1.60^{a}	0.98^{ab}	0.303	0.972	0.023	0.176
	OCLN	0.96	1.29	1.09	1.42	0.87	0.286	0.169	0.176	0.297
	TLR2	2.34^{A}	0.63^{B}	0.77	2.34	1.35	0.649	0.004	0.069	0.107
	TLR4	1.91	1.05	1.28	1.18	1.97	0.538	0.061	0.293	0.082
	NF-κB	1.04	1.21	0.87	1.23	1.28	0.310	0.506	0.363	0.968
	MUC2	1.17	1.22	0.87^{B}	1.74^{A}	0.96^{B}	0.212	0.770	0.001	0.486
	sIgA	1.51^{A}	0.79^{B}	0.85	1.38	1.23	0.269	0.003	0.154	0.021
Cecal										
	ZO-1	1.57	1.08	1.19	1.76	1.03	0.330	0.083	0.085	0.488
	ZO-2	1.23	1.39	1.24	1.26	1.42	0.360	0.599	0.861	0.716
	CLDN1	1.69^{A}	1.04^{B}	1.37	1.62	1.10	0.353	0.035	0.361	0.077
	CLDN5	1.40	1.47	1.80	1.55	0.95	0.492	0.866	0.227	0.954
	OCLN	1.56	1.25	1.50	1.84	0.88	0.430	0.378	0.098	0.209
	TLR2	2.84^{A}	0.93^{B}	2.44^{a}	2.40^{a}	0.82^{b}	0.618	0.001	0.022	0.399
	TLR4	1.18	1.27	1.37	0.82	1.48	0.419	0.810	0.256	0.973
	NF-κB	1.34	1.29	1.18	1.87	0.90	0.420	0.866	0.078	0.973
	MUC2	1.25	1.22	1.45	1.11	1.15	0.487	0.938	0.749	0.126
	sIgA	2.25^{A}	1.21^{B}	1.57	2.00	1.61	0.366	0.002	0.441	0.291

PFA = phytogenic feed additive; SEM = pooled standard error of means.
$^{a, b}$ Within a row, means with different superscripts differ at $P < 0.05$.
$^{A, B}$ Within a row, means with different superscripts differ at $P < 0.01$.
[1] Relative expression ratios of target genes were calculated according to Pfaffl et al. (2001) using GAPDH as reference gene.
[2] Basal diets based on maize (M) or wheat (W). Data shown per cereal type represent treatment means from 15 broilers (e.g. 5 from treatment M0 + 5 from treatment M100 + 5 from treatment M150).
[3] Phytogenic supplementation (0, 100 and 150 mg/kg diet). Data shown for PFA represent means from 10 replicate pens (e.g. 5 from treatment M0 + 5 from treatment W0).

and *Enterobacteriaceae* (Kaldhusdal and Hofshagen, 1992; Choct et al., 1996; Rodriguez et al., 2012).

On the other hand, irrespective of cereal type, PFA inclusion had no direct significant effect on any of the gut microbiota constituents examined. However, PFA inclusion in broiler diets has been reported to reduce pathogenic members such as *E. coli* (Cho et al., 2014; Hashemipour et al., 2016), *Salmonella* (Pathak et al., 2016) and/or even enhance beneficial members such as *Lactobacillus* and *Bifidobacterium* (Mountzouris et al., 2011; Franciosini et al., 2015; Hashemipour et al., 2016). There are also reports where no effects on gut commensal bacteria were shown (Hong et al., 2012; Pathak et al., 2016), in line with the findings in this work. Important factors such as PFA composition, PFA inclusion level(s), farm hygiene status as well as the analytical approach employed for gut microbiology could provide reasonable explanations for the lack of effects on gut microbiota composition. In this respect, the possibility for wider changes on gut microbiota composition, not accounted for by the microbial members determined in this study, cannot be excluded.

From another perspective, VFA as the major products of microbial metabolism are considered as key indicators of microbial metabolic activity (Cummings and Macfarlane, 1991; Cao et al., 2010; Hashemipour et al., 2016). Among the major VFA properties are their beneficial implications for energy salvage by the host (Cummings and Macfarlane, 1991), their uptake and utilization as the preferred energy source by the colonic epithelial cells (Cao et al., 2010; Svihus et al., 2013) and last but not least strong antimicrobial properties (Van der Wielen et al., 2000). Diet is known to affect the intensity and the pattern of microbial fermentation in the gut. Fermentation intensity is linked with the total VFA concentration, whereas fermentation pattern is illustrated by the molar ratios of VFA constituent components (Mountzouris et al., 2007, 2015; Cross et al., 2011).

In this study, the combination of cereal type with PFA inclusion level had a significant impact on the pattern of fermentation process at ileal and cecal level as shown by the significant interactions regarding the molar ratios of ileal propionic as well as branched-VFA at ileum and ceca. In addition, cereal type effects on VFA were confined at ceca, whereas PFA inclusion affected the intensity and pattern of VFA both at ileum and ceca. The ceca are known as the major site of microbial fermentation in the avian gut (Svihus et al., 2013) and this explains the more than 10 folds higher total VFA concentration in ceca compared with that in the ileum. In turn, the higher total VFA determined for wheat compared with maize may be explained by wheat's intrinsic properties that may result in more fermentable substrates reaching ceca (Hubener et al., 2002; Hashemipour et al., 2016). The PFA inclusion level had a fermentation stimulating effect at ileum and a retarding effect in ceca. Most of the PFA active components are known to be absorbed in the proximal gut (Lee et al., 2004; Michiels et al., 2008) and in this respect potential PFA direct effects at ileum would be expected to be small. However, in ceca due to the physiological reflux of urinary components (Sacranie et al., 2012), it could be possible that urinary excreted PFA and their metabolites are refluxed back in the ceca. It could then be postulated that the lower VFA concentration with increasing PFA level could be due to a generalized PFA antimicrobial effect (Cho et al., 2014; Franciosini et al., 2015). Therefore, despite the absence of significant compositional changes in the determined cecal microbiota constituents, the VFA components could have resulted in less microbial activity in this work.

The changes in the VFA molar pattern as the ones seen in this work may depend on the microbiota composition (Cao et al., 2010) as well as on the amount and type of feed substrates such as the non-starch carbohydrate fraction of wheat reaching the ceca (Cummings and Macfarlane, 1991; Svihus et al., 2013). In addition, according to the type of the cereal of the BD, wheat diets could

increase cecal concentrations of acetic and butyric acids, whereas maize diets exhibited higher concentrations of propionic, valeric, and isovaleric acids (Kiarie et al., 2014).

In chickens, *TLR* signaling ultimately results in the activation of *NF-κB* and the subsequent production of an inflammatory response (Keestra et al., 2013). As a result, down-regulation of *TLR* could be essential for limiting inflammation (Kawai and Akira, 2007). In the present study, PFA administration down-regulated cecal mucosa *TLR2* expression at broilers supplemented PFA at 150 mg/kg diet. Down-regulation of cecal *TLR* by PFA supplementation has been also shown by other studies (Lu et al., 2014; Du et al., 2016). A possible PFA mode of action is the inhibition of *TLR* activation by targeting directly the receptors or the specific downstream signaling molecules (Lillehoj and Lee, 2012). On the other hand, *TLR* expression was not affected in broilers fed cereals other than maize under coccidial challenge (Chen et al., 2015), suggesting that other microbiota members could be implicated in triggering changes in *TLR* signaling. For example, the fact that ileal and cecal mucosa *TLR2* expression levels were lower in broilers fed wheat diets in this study may be linked with the respective higher *Lactobacillus* levels in ileal mucosa and the lower cecal digesta total bacteria and *Clostridia* levels.

The maintenance of gut barrier is essential for gut function and health (Suzuki and Hara, 2011; Du et al., 2016). Tight junction (TJ) proteins such as occludin (*OCLN*), claudins (*CLDNs*), and zonula occludens (*ZO*) act as a barrier preventing paracellular permeability (Hu et al., 2012; Liu et al., 2012; Song et al., 2014). In addition, other intestinal elements such as mucin and *sIgA* provide protection against luminal threats (Tsirtsikos et al., 2012; Du et al., 2016). In this work, from the gut barrier genes studied, a limited interaction of cereal type with PFA administration were shown only for the *sIgA* m-RNA transcripts at the ileal mucosa. Interestingly, the rest of the results suggested a different intestinal homeostasis management depending on the cereal used. For example, given the higher microbiota populations in maize-fed birds, it is likely that the maize-fed birds faced a higher microbiota challenge and responded by increasing *TLR2* expression as well as *sIgA* and *CLDN1* compared with wheat-fed birds. On the other hand, an explanation for the higher ileal mucosa *ZO-2* in broilers fed wheat compared with maize could be to counteract probable undesirable intestinal effects such as increased digesta viscosity caused by the higher soluble NSPs levels of wheat (Hubener et al., 2002; Liu et al., 2012; Lee et al., 2017).

Furthermore, irrespective of cereal type, PFA inclusion level increased the expressions of ileal *CLDN5* and *MUC2* genes conferring additional protection to the gut barrier. It is known that the enhancement of TJ assembly by PFA supplementation could lead to a promotion of intestinal barrier integrity (Suzuki and Hara, 2011; Zou et al., 2016).

5. Conclusion

In conclusion, this study has provided further evidence that cereal type and PFA inclusion independently and in combination affected broiler gut microbiota composition (e.g. *Lactobacillus* spp. *Bifidobacterium* spp. and *Clostridia* clusters I, IV and XIVa) and metabolic activity (e.g. total VFA, acetic acid, butyric acid, b-VFA and o-VFA) as well as the expression of critical gut barrier genes (e.g. *ZO-2*, *CLDN5* and *MUC2*) including *TLR2* a well-known (Keestra et al., 2013) essential signaling component for immune homeostasis. Therefore, the baseline knowledge generated in this study under non-pathogenic conditions merits further exploitation under stress-challenge conditions in future studies so as to further confirm potential benefits for gut health.

Conflict of interest

None.

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