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Original Research Article

# Diet supplementation with an organic acids-based formulation affects gut microbiota and expression of gut barrier genes in broilers

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ABSTRACT

This study was designed to study the effect of diet supplementation with an organic acids-based formulation (OABF) on luminal- and mucosa-associated bacteria, concentration of volatile fatty acids (VFA), microbial glycolytic enzyme activity and expression of mucin 2 (*MUC2*), immunoglobulin A (*IgA*) and tight junction protein, i.e., zonula occludens-1 (*ZO1*), zonula occludens-2 (*ZO2*), claudin-1 (*CLDN1*), claudin-5 (*CLDN5*) and occludin (*OCLN*), genes at the ileal and cecal level. A 2 × 2 factorial design was used having OABF inclusion and avilamycin as main factors. Subsequently, 544 day-old male Cobb broilers were allocated in the following 4 treatments, each with 8 replicates: no additions (CON), 1 g OABF/kg diet (OA), 2.5 mg avilamycin/kg diet (AV) and combination of OA and AV (OAAV). The trial lasted for 42 days. In the ileum, OAAV resulted in lower mucosa-associated total bacteria levels ($P_{O \times A} = 0.028$) compared with AV. In addition, ileal digesta levels of *Clostridium perfringens* subgroup were decreased by avilamycin ($P_A = 0.045$). Inclusion of OABF stimulated the activity of microbial glycolytic enzymes, whereas avilamycin resulted in lower acetate ($P_A = 0.021$) and higher butyrate ($P_A = 0.010$) molar ratios. Expression of *ZO1* and *CLDN5* was down-regulated by both OABF ($P_O = 0.016$ and $P_O = 0.003$, respectively) and avilamycin ($P_A = 0.016$ and $P_A = 0.001$, respectively). In addition, *CLDN1* was down-regulated in AV compared with CON ($P_{O \times A} = 0.012$). Furthermore, OABF down-regulated *MUC2* ($P_O = 0.027$), whereas avilamycin down-regulated nuclear factor kappa B subunit 1 (*NFKB1*) ($P_A = 0.024$), toll-like receptor 2 family member B (*TLR2B*) ($P_A = 0.011$) and toll-like receptor 4 (*TLR4*) ($P_A = 0.014$) expression. In the ceca, OABF inclusion increased digesta levels of *Clostridium coccoides* ($P_O = 0.018$) and *Clostridium leptum* ($P_O = 0.040$) subgroups, while it up-regulated *MUC2* expression ($P_O = 0.014$). Avilamycin ($P_A = 0.044$) and interaction ($P_{O \times A} < 0.001$) effects for *IgA* expression were noted, with CON having higher *IgA* expression compared with AV. In conclusion, new findings regarding OABF inclusion effects on an array of relevant biomarkers for broiler gut ecology have been reported and discussed in parallel with avilamycin effects used as a positive control. This new knowledge is expected to provide a response baseline for follow up trials under various stress and challenge conditions.



## 1. Introduction

Optimization of animal performance is the main goal of modern farming. Until 2006, the use of antibiotic growth promoters (AGP) in the European Union was a key element for improving animal production. Since then, worldwide scientific research has been exploring equally effective alternatives that will maintain animal health, improve animal performance and have no negative impact on animal welfare and consumer health (Shanmugavelu et al., 2006; Goodarzi Boroojeni et al., 2014).

However, the missing knowledge about the exact mechanism of how AGP enhance animal growth (Niewold, 2007) in combination

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with the largely unknown complexity of the gastrointestinal ecosystem, makes the development of effective alternatives not straightforward. The ongoing elucidation of the extensive interactions between the poultry host and its gut microbiome such as exchange of nutrients, modulation of host gut morphology, physiology and immunity is required in order to develop future powerful dietary strategies (Choct 2009; Pan and Yu, 2014).

So far, among various substances being researched for their effects on broiler nutrition, organic acids- based formulations (OABF) have received significant attention (Huyghebaert et al., 2011). Organic acids have long being utilized in the food industry due to their direct and indirect antimicrobial activity (Van Immerseel et al., 2006; Mani-Lopez et al., 2012). Dietary inclusion of an OABF in poultry feed has been shown to modulate gut luminal microbiota composition (Nava et al., 2009; Czerwiński et al., 2010, 2012; Sun et al., 2013). The incorporation of OABF in broiler feed has been shown to exhibit a positive response in performance (Garcia et al., 2007; Abdel-Fattah et al., 2008; Chowdhury et al., 2009; Samanta et al., 2010; Palamidi et al., 2016), but the exact mechanism behind their growth promoting ability has not been fully elucidated. In addition, direct comparisons of OABF effects as alternatives to AGP are scarce.

From our previous research, it has been shown that inclusion of an OABF consisting of selected organic acids (i.e., formic, propionic and acetic acid) and their salts affected broiler growth performance, nutrient digestibility and energy retention in a beneficial way (Palamidi et al., 2016). The aim of this work was to generate new knowledge on OABF inclusion effects that could further support zootechnical performance findings by focusing in the study of key gut ecosystem elements. In particular, the study aimed to determine changes in luminal- and mucosa associated bacterial groups, concentration of volatile fatty acids (VFA), activity of microbial glycolytic enzymes and gene expression of several gut barrier and health biomarkers at ileal and cecal level.

## 2. Materials and methods

### 2.1. Birds and experimental treatments

This study forms part of our previous research work (Palamidi et al., 2016) and in order to avoid excessive repetition, a brief description of the experimental treatments is given below. A total of 544 day-old male Cobb broilers vaccinated for Marek's disease, infectious bronchitis and Newcastle disease were acquired from a local hatchery. Birds were arranged according to a 2 × 2 factorial design in 4 treatments, with 8 ($n = 8$) replicate pens of 17 chicks per treatment for a 42-d study. All experimental treatments received a corn-soybean basal diet formulated for starter (1 to 14 d), grower (15 to 28 d) and finisher (29 to 42 d) growth periods. The calculated chemical composition per kg of basal diets were: starter ($AME_n$ 12.5 MJ; crude protein 210 g; lysine 12 g; calcium 10 g and available phosphorus 5 g), grower ($AME_n$ 12.9 MJ; crude protein 190 g; lysine 11 g; calcium 9.6 g and available phosphorus 4.8 g) and finisher ($AME_n$ 13.3 MJ; crude protein 180 g; lysine 10.5 g; calcium 9 g and available phosphorus 4.5 g). Depending on the addition of OABF and/or avilamycin used as a model AGP, experimental treatments were classified as: no additions (CON), 1 g OABF/kg diet (OA), avilamycin 2.5 mg active components/kg diet (AV) and combination of OA + AV (OAAV). There was a coccidiostat addition in the starter and grower basal diets. Diets and water availability were *ad libitum* for the whole experiment. The OABF (Biotronic Top3, Biomin GmbH, Herzogenburg, Austria) consisted of selected organic acids (i.e., formic, propionic and acetic acid) and their salts at 394 g/kg, flavoring components (i.e., cinnamaldehyde and a permeabilizing substance) and carrier.

Birds were reared in an experimental facility designed for broilers and constructed according to the international standards for sterile rooms ISO 14644-1 and F.S. 290E (ClimaThermica Ltd, Athens, Greece) fitted with airlock doors and absolute air filters in all air inlets and outlets. After the trial, all birds were euthanized and incinerated. 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.

### 2.2. Sampling and processing

At the end (42 d) of the experiment, 8 birds per treatment (i.e., one bird/cage) were randomly selected and euthanized, then dissected and relevant samples taken. In particular, broilers were opened, under aseptic conditions and the whole ileum and the 2 ceca were removed. All samples were immediately snap-frozen in liquid nitrogen followed by storage in −80 °C until further analysis.

#### 2.2.1. Sample preparation for microbiological analysis

For the determination of luminal- and mucosa-associated microbiota composition and metabolic activity (i.e., VFA and glycolytic enzymes), a 15-cm segment of broiler ileum and one of the ceca were used.

Ileal and cecal segments were thawed on ice and opened longitudinally. Firstly, digesta content was removed carefully and then in order to remove remaining digesta and bacteria not attached to the gut mucosa, each gut segment was washed 3 times in ice cold saline by gentle agitation. Subsequently, mucosa attached bacteria were removed from the gut mucosa following a protocol of 3 × 1 min vigorous hand shaking washes (15 mL) in saline containing 0.1% (wt/wt) Tween 80, according to Li et al. (2003). Finally, the washes were pooled and centrifuged at 10,000 × *g* for 30 min at 4 °C to precipitate cells (cell pellet).

#### 2.2.2. Sample preparation for gene expression studies

For the relative expression of mucin 2 (*MUC2*), immunoglobulin A (*IgA*), zonula occludens-1 (*ZO1*), zonula occludens-2 (*ZO2*), claudin-1 (*CLDN1*), claudin-5 (*CLDN5*), occludin (*OCLN*), nuclear factor kappa B subunit 1(*NFKB1*), toll-like receptor 2 family member B (*TLR2B*) and toll-like receptor 4 (*TLR4*), a 10-cm segment of broiler ileum and the second entire cecum were used.

Ileal and cecal segments were thawed on ice and opened longitudinally. Digesta contents were removed carefully and each gut segment was then washed 3 times in ice cold saline by gentle agitation. Subsequently, the cleaned gut mucosa was further washed with ice cold ethylene diamine tetraacetic acid (0.1 mol/L EDTA, pH 7.2). Finally, mucosal scrapings were cautiously obtained with the help of a microscope glass slide.

### 2.3. DNA extraction

Ileal, cecal digesta and cell pellets from ileum and caecum were used for DNA extraction using a suitable commercial kit (PSP Spin Stool DNA Kit, Stratec Molecular GmbH, Berlin, Germany). The lysis protocol was optimised by incorporating an additional 30 min lysozyme and a 15 min RNase digestion step. For each sample, the purified 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.

### 2.4. Quantitative real time PCR

To quantify total bacteria (domain bacteria), *Lactobacillus* spp., *Escherichia coli*, *Bifidobacterium* spp., *Bacteroides* spp., *Clostridium perfringens* subgroup (*Clostridium* cluster I), *Clostridium leptum* subgroup (*Clostridium* cluster IV) and *Clostridium coccoides* subgroup (*Clostridium* cluster XIVa), suitable primers were used targeting the 16S rRNA gene (Table 1). Primer specificity was confirmed using BLAST (NCBI) and PROBE MATCH program (Ribosomal Database Project II; Cole et al., 2014).

Real time PCR was performed in microplates with the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were made at a 15 μL final volume and consisted of a 7.5 μL 2 × Green Dye master mix (Rovalab GmbH, Teltow, Germany), forward and reverse primers each at final concentration of 300 to 450 nmol/L, 0.75 μL of bovine serum albumin (20 μg/mL), 0.15 μL passive ROX reference dye (50 nmol/L final concentration) and 2 μL of DNA template (20 ng sample DNA/reaction). The reactions were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, primer specific annealing temperature for 60 s, then 72 °C for 60 s. Target amplification was followed by a melt curve analysis to determine the reaction specificity. Each sample was measured in duplicates. Depending on whether the sample was from mucosa or luminal digesta, results were expressed as log cells/g mucosa-associated cell pellet or as log cells/g wet digesta contents, respectively.

### 2.5. Bacterial strains and calibration curves

Reference bacterial strains that were used to control the specificity of the primers and to construct standard curves are shown on Table 2. Each of the reference strains was cultured on selective broth under suitable conditions. Bacterial genomic DNA from each culture was extracted using PSP Spin Stool DNA Kit (Stratec Molecular GmbH, Berlin, Germany).

For the quantification of bacterial species and groups, a quantification method similar to the one described by Joly et al. (2006) was used. In more detail, an appropriate standard curve using 10-fold serial dilutions of known concentration of genomic DNA was included on each 96-well plate. The number of genome copies, from each bacterial species in the initial purified DNA solution used to construct the standard curves, was calculated by assuming an average molecular mass of 660 Da for 1 bp of double-stranded DNA and using the following equation: Number of genome copies = Quantity of DNA (fg)/Mean mass of the corresponding genome (fg). The number of genome copies corresponds to an equal amount of bacterial cells. Genome sizes for all bacteria species and groups used in this study are presented in Table 2.

### 2.6. RNA isolation and reverse transcription to cDNA

Extraction of total RNA from ileal and cecal mucosal scrapings was performed using Trifast Reagent (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer's protocol. RNA quantity was determined by spectrophotometry (NanoDrop-1000, Thermo Fisher Scientific, Waltham, UK). Prior to 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 DNAse buffer (10 × ) 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, Japan) according to the manufacturer's recommendations. cDNAs were stored at −20 °C.

### 2.7. Quantitative polymerase chain reaction (qPCR)

The ileal and cecal mRNA expression of *MUC2* and *IgA*, *TLR2* and *TLR4*, intestinal tight junctions (*CLDN1*, *CLDN2*, *CLDN5* and *OCLN*) and *NFKB1* were detected using quantitative real time PCR SaCycler-96 (Sacace Biotechnologies Srl) with KAPA SYBR Fast qPCR Kits (KAPA Biosystems, Wilmington, MA, USA). The primer sequences used for real-time PCR are listed in Table 3. Primers not originating from scientific literature were designed with the Perl-Primer program v.1.1.19 (Marshall, 2004) using the GenBank sequences. Primer specificity and efficiency were determined by using pooled samples.

Each reaction contained 5 ng RNA equivalents as well as 200 to 300 nmol/L of forward and reverse primers for each gene. The reactions were incubated at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 59 or 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 glyceraldehyde 3 phophate dehydrogenase (*GAPDH*) as a reference gene.

### 2.8. Digesta volatile fatty acid concentration

For the determination of ileal or cecal VFA concentration, digesta were homogenized following a 10-fold dilution (i.e., 10% wt/vol) in

**Table 1**
Primers targeting 16S rRNA gene used for determination of luminal- and mucosa-associated microbiota composition by real time PCR.

| Target group or organism | Sequence (5′ to 3′) | Annealing temperature, °C | Reference |
|---|---|---|---|
| All bacteria (domain bacteria) | F: ACTCCTACGGGAGGCAGCAG<br>R: ATTACCGCGGCTGCTGG | 60 | Clifford et al., 2012 |
| *Bacteroides* spp. | F: GAGAGGAAGGTCCCCCAC<br>R: CGCTACTTGGCTGGTTCAG | 58 | Peinado et al., 2013 |
| *Lactobacillus* spp. | F: GAGGCAGCAGTAGGGAATCTTC<br>R:GGCCAGTTACTACCTCTATCCTTCTTC | 60 | Delroisse et al., 2008;<br>Peinado et al., 2013 |
| *Bifidobacterium* spp. | F: CGCGTCYGGTGTGAAAG<br>R: CCCCACATCCAGCATCCA | 58 | Delroisse et al., 2008;<br>Peinado et al., 2013 |
| *Escherichia coli* | F: CATGCCGCGTGTATGAAGAA<br>R:GGGTAACGTCAATGAGCAAAGG | 60 | Silkie and Nelson, 2009 |
| *Clostridium perfringens* subgroup (*Clostridium* cluster I) | F: TACCHRAGGAGGAAGCCAC<br>R:GTTCTTCCTAATCTCTACGCAT | 56 | Goodarzi Boroojeni et al., 2014 |
| *Clostridium leptum* subgroup (*Clostridium* cluster IV) | F: GCACAAGCAGTGGAGT<br>R: CTTCCTCCGTTTTGTCAA | 52 | Matsuki et al., 2004 |
| *Clostridium coccoides* subgroup (*Clostridium* cluster XIVa) | F: ACTCCTACGGGAGGCAGC<br>R:CTTCTTAGTCAGGTACCGTCAT | 60 | Schwiertz et al., 2010 |

**Table 2**
Reference strains and genome sizes.

| Reference strains | Target bacterial group(s) | NCBI reference sequence | Genome size, Mbp |
|---|---|---|---|
| *Escherichia coli* ATCC 25922 | *Escherichia* sp. & domain bacteria | NZ_CP009072.1 | 5.13 |
| *Bacteroides vulgatus* ATCC 8482 | *Bacteroides* spp. | NC_009614.1 | 5.16 |
| *Lactobacillus acidophilus* ATCC 314 | *Lactobacillus* spp. | NC_006814.3 | 1.99 |
| *Bifidobacterium animalis* subsp. *animalis* ATCC 25527 | *Bifidobacterium* spp. | NC_017834.1 | 1.93 |
| *Clostridium perfringens* ATCC 13124 | *C. perfringens* subgroup (*Clostridium* cluster I) | NC_008261.1 | 3.26 |
| *Clostridium leptum* DSM 753 | *C. leptum* subgroup (*Clostridium* cluster IV) | NZ_ABCB00000000.2 | 3.27 |
| *Clostridium clostridioforme* DSM933 | *C. coccoides* subgroup (*Clostridium* cluster XIVa) | NZ_FOOJ00000000.1 | 5.47 |

sterile ice-cold phosphate buffered saline (0.1 mol/L, pH 7.0). Digesta homogenates were subsequently centrifuged 12,000 × *g* for 10 min at 4 °C and the resulting supernatants were stored at −80 °C until their analysis by capillary gas chromatography (GC) using an Agilent 6890 GC System, equipped with a 30 m × 0.25 mm i.d. Nukol column (Supelco, Sigma-Adrich, St Louis, MO, USA) and a flame ionisation detector (FID). The analysis was isothermal (185 °C) and the temperatures of the injector and FID were set at 185 and 200 °C, respectively, as previously described (Mountzouris et al., 2014).

The VFA determined were acetic, propionic, isobutyric, butyric, isovaleric, valeric, isohexanoic, hexanoic and heptanoic acids. Results were expressed as mmol/kg wet digesta for total VFA and as molar ratios (% of total VFA) for acetic, propionic, butyric, branched VFA (b-VFA; sum of isobutyric, isovaleric and isohexanoic acids) and other VFA (o-VFA; sum of valeric, hexanoic and heptanoic acids).

### 2.9. Digesta activity of microbial glycolytic enzymes

Microbial glycolytic activities of α-glucosidase, β-glucosidase, β-galactosidase and β-glucuronidase enzymes were determined through the rate of release of p-nitrophenol (pNP) from the respective p-nitrophenylglucoside substrates namely α-glucoside (1 mmol/L), β-glucoside (1 mmol/L), β-galactoside (2 mmol/L) and β-glucuronide (1 mmol/L) according to Mountzouris et al. (2007). Briefly, 1 volume of diluted digesta supernatants (see above) in sterile ice-cold PBS was reacted with 4 volumes (1:4) of the appropriate p-nitrophenylglucoside substrate prepared in sterile PBS (0.1 mol/L, pH 7.0) that had been pre-equilibrated to the reaction temperature. The reaction time was 25 min at 37 °C. The reaction was stopped by the addition of 10 volumes of ice-cold $Na_2CO_3$ (1 mol/L) and absorbance measured at 405 nm. All enzyme activities were calculated using a standard curve for pNP and were expressed as μmol of pNP released per minute per digesta soluble protein.

### 2.10. Statistical analysis

Experimental data on luminal- and mucosa-associated microbiota, microbial glycolytic enzyme activity, VFA and the relative quantification of genes of interest were based on individual broilers. All data were tested for normality using the Kolmogorov–Smirnov test and found to be normally distributed. Subsequently, data were analyzed with the general linear model (GLM) – general factorial ANOVA procedure using OABF (No/Yes) and avilamycin (No/Yes) as fixed factors. Probability values of equal or less than 0.05 ($P \leq 0.05$) were considered significant. Statistical significant effects were further analyzed and treatment means were compared using Tukey HSD test using the SPSS for Windows statistical package program, version 8.0.0 (SPSS, Chicago, IL, USA).

## 3. Results

### 3.1. Microbiota composition

#### 3.1.1. Ileum

The inclusion of OABF or avilamycin had no impact ($P > 0.05$) on the concentration of total bacteria, *Bacteroides* spp., *Lactobacillus*

**Table 3**
Oligonucleotide primers used for quantitative RT-PCR.

| Target | Primer sequence (5′ to 3′) | Annealing temperature, °C | PCR product size, bp | GenBank accession No. |
|---|---|---|---|---|
| *GAPDH* | F: GCTGAATGGGAAGCTTACTG<br>R: AAGGTGGAGGAATGGCTG | 60 | 216 | NM_204305.1 |
| *MUC2* | F:TCACCCTGCATGGATACTTGCTCA<br>R:TGTCCATCTGCCTGAATCACAGGT | 62 | 228 | NM_001318434.1 |
| *IgA* | F:GTCACCGTCACCTGGACTACA<br>R:ACCGATGGTCTCCTTCACATC | 60 | 192 | S40610 |
| *ZO1* | F:TAAAGCCATTCCTGTAAGCC<br>R:GTTTCACCTTTCTCTTTGTCC | 60 | 243 | XM_015278975.1 |
| *ZO2* | F:GTTTCACCTTTCTCTTTGTCC<br>R:TAAAGCCATTCCTGTAAGCC | 60 | 239 | NM_204918.1 |
| *CLDN1* | F:CTGATTGCTTCCAACCAG<br>R:CAGGTCAAACAGAGGTACAAG | 59 | 140 | NM_001013611.2 |
| *CLDN5* | F:CATCACTTCTCCTTCGTCAGC<br>R:GCACAAAGATCTCCCAGGTC | 59 | 111 | NM_204201.1 |
| *OCLN* | F:TCATCGCCTCCATCGTCTAC<br>R:TCTTACTGCGCGTCTTCTGG | 62 | 240 | NM_205128.1 |
| *NFKB1* | F: TGTGGTTGTCAGGATGGTC<br>R: GGTCTGGTAAAGGTCATTTCTC | 62 | 273 | NM_205134 |
| *TLR2B* | F:CTTGGAGATCAGAGTTTGGA<br>R:ATTTGGGAATTTGAGTGCTG | 62 | 238 | NM_001161650.1 |
| *TLR4* | F: GTCTCTCCTTCCTTACCTGCTGTTC<br>R:AGGAGGAGAAAGACAGGGTAGGTG | 65 | 187 | NM_001030693.1 |

*GAPDH* = glyceraldehyde 3 phophate dehydrogenase; *MUC2* = mucin 2; *IgA* = immunoglobulin A; *ZO1* = zonula occludens-1; *ZO2* = zonula occludens-2; *CLDN1* = claudin-1; *CLDN5* = claudin-5; *CLDN* = occludin; *NFKB1* = nuclear factor kappa B subunit 1; *TLR2B* = toll-like receptor 2 family member B; *TLR4* = toll-like receptor 4.

spp., *E. coli*, *C. leptum* subgroup and *C. coccoides* subgroup in the ileal contents (Table 4). However, the concentration of *C. perfringens* subgroup in the ileum was significantly decreased ($P_A = 0.045$) by avilamycin inclusion.

A significant OABF × avilamycin interaction ($P_{O \times A} = 0.028$) was noted for the concentration of ileal mucosa-associated total bacteria. In particular, total bacteria counts were lower in OAAV compared with AV (Table 5).

### 3.1.2. Cecum

Inclusion of OABF resulted in significantly increased levels of *C. leptum* subgroup and *C. coccoides* subgroup ($P_O = 0.018$, $P_O = 0.040$, respectively) in the cecal digesta (Table 6). In contrast, avilamycin did not affect ($P > 0.05$) any of the determined microbiota components. In addition, cecal mucosa-associated bacterial populations were not affected ($P > 0.05$) by OABF or avilamycin inclusion (Table 7).

## 3.2. Microbial glycolytic activity

### 3.2.1. Ileum

The inclusion of OABF resulted in increased activities of α-glucosidase ($P_O = 0.028$), β-glucosidase ($P_O = 0.014$), α-galactosidase ($P_O = 0.021$) and β-glucuronidase ($P_O = 0.005$) in ileal digesta (Table 8). Avilamycin inclusion did not affect any of the microbial glycolytic activities examined.

### 3.2.2. Caecum

In the cecal digesta, except for β-galactosidase that was significantly decreased ($P_A = 0.031$) by avilamycin addition, no other changes regarding the microbial glycolytic activities were seen (Table 9).

## 3.3. Volatile fatty acid concentration

### 3.3.1. Ileum

Total VFA concentration in the ileal digesta was not affected by OABF or avilamycin addition (Table 10). Regarding the molar ratios of individual VFA, avilamycin had an effect on acetic acid and butyric acid. In particular, avilamycin inclusion resulted in a lower molar ratio of acetic acid ($P_A = 0.021$) and a higher molar ratio of butyric acid ($P_A = 0.010$) compared with the non-avilamycin supplemented treatments.

**Table 5**
Effect of dietary inclusion of an organic acids-based formulation (OABF) and avilamycin dietary inclusion on ileal mucosa-associated bacteria (log cells/g mucosa-associated cell pellet) of 42-day-old broilers.

| Item | | Total bacteria | *Lactobacillus* spp. | *Clostridium coccoides* subgroup |
|---|---|---|---|---|
| Main effect | | | | |
| OABF [1] | No | 7.19 | 5.81 | 6.09 |
| | Yes | 7.09 | 5.75 | 6.07 |
| Avilamycin[2] | No | 7.14 | 5.85 | 6.15 |
| | Yes | 7.14 | 5.72 | 6.00 |
| Treatments[3] | | | | |
| CON | | 7.07[ab] | 5.73 | 5.96 |
| OA | | 7.22[ab] | 5.96 | 6.34 |
| OAAV | | 6.97[b] | 5.54 | 5.79 |
| AV | | 7.31[a] | 5.90 | 6.21 |
| Statistics | | | | |
| SEM[4] | | 0.106 | 0.184 | 0.233 |
| $P_O$ | | 0.368 | 0.731 | 0.931 |
| $P_A$ | | 0.956 | 0.485 | 0.525 |
| $P_{O \times A}$ | | 0.028 | 0.124 | 0.096 |

[a, b] Interaction means within the same column differ significantly ($P \leq 0.05$).
[1] No: no OABF addition; Yes: addition of 1 g OABF/kg diet.
[2] No: no avilamycin addition; Yes: addition of 2.5 mg of avilamycin/kg diet.
[3] Interaction means from 8 replicate pens per treatment. Treatments include: control − no additions (CON), 1 g OABF/kg diet (OA), avilamycin 2.5 mg active components/kg diet (AV) and combination of OA + AV (OAAV).
[4] Pooled standard error of means.

### 3.3.2. Caecum

From the VFA determined in the cecal digesta, inclusion of avilamycin significantly increased ($P_A = 0.043$) the molar ratio of o-VFA (Table 11). There were no other VFA changes by neither OABF nor avilamycin inclusion.

## 3.4. Gene expression of intestinal mucosal barrier proteins

### 3.4.1. Ileum

Gene expression of *MUC2, IgA, ZO1, ZO2, CLDN1, CLDN5, OCLN, NFKB1, TLR2b* and *TLR4* results are shown in Table 12. Regarding factor main effects, supplementation with OABF significantly

**Table 4**
Effect of dietary inclusion of an organic acids-based formulation (OABF) and avilamycin on ileal digesta microbiota (log cells/g wet digesta content) of 42-day-old broilers.

| Item | | Total bacteria | *Bacteroides* spp. | *Lactobacillus* spp. | *Escherichia coli* | *Clostridium perfringens* subgroup | *Clostridium leptum* subgroup | *Clostridium coccoides* subgroup |
|---|---|---|---|---|---|---|---|---|
| Main effect | | | | | | | | |
| OABF[1] | No | 8.12 | 5.76 | 7.43 | 5.90 | 6.42 | 5.44 | 6.71 |
| | Yes | 8.13 | 5.92 | 7.46 | 6.10 | 6.68 | 5.48 | 6.94 |
| Avilamycin[2] | No | 8.11 | 5.98 | 7.58 | 6.23 | 6.88 | 5.52 | 6.88 |
| | Yes | 8.14 | 5.69 | 7.30 | 5.77 | 6.22 | 5.41 | 6.77 |
| Treatments[3] | | | | | | | | |
| CON | | 8.05 | 5.84 | 7.47 | 6.21 | 6.85 | 5.45 | 6.88 |
| OA | | 8.17 | 6.12 | 7.70 | 6.25 | 6.90 | 5.58 | 6.88 |
| OAAV | | 8.09 | 5.72 | 7.22 | 5.95 | 6.45 | 5.38 | 7.00 |
| AV | | 8.19 | 5.67 | 7.38 | 5.58 | 5.98 | 5.44 | 6.54 |
| Statistics | | | | | | | | |
| SEM[4] | | 0.116 | 0.259 | 0.242 | 0.287 | 0.314 | 0.138 | 0.132 |
| $P_O$ | | 0.958 | 0.538 | 0.894 | 0.491 | 0.421 | 0.759 | 0.095 |
| $P_A$ | | 0.827 | 0.271 | 0.258 | 0.116 | 0.045 | 0.440 | 0.414 |
| $P_{O \times A}$ | | 0.363 | 0.668 | 0.429 | 0.563 | 0.509 | 0.483 | 0.099 |

[1] No: no OABF addition; Yes: addition of 1 g OABF/kg diet.
[2] No: no avilamycin addition; Yes: addition of 2.5 mg of avilamycin/kg diet.
[3] Interaction means from 8 replicate pens per treatment. Treatments include: control − no additions (CON), 1 g OABF/kg diet (OA), avilamycin 2.5 mg active components/kg diet (AV) and combination of OA + AV (OAAV).
[4] Pooled standard error of means.

**Table 6**
Effect of dietary inclusion of an organic acids-based formulation (OABF) and avilamycin dietary inclusion on cecal digesta microbiota (log cells/g wet digesta content) of 42-day-old broilers.

| Item | | Total bacteria | *Bacteroides* spp. | *Lactobacillus* spp. | *Bifidobacterium* spp. | *Escherichia coli* | *Clostridium perfringens* subgroup | *Clostridium leptum* subgroup | *Clostridium coccoides* subgroup |
|---|---|---|---|---|---|---|---|---|---|
| Main effect | | | | | | | | | |
| OABF[1] | No | 10.12 | 9.52 | 7.08 | 5.37 | 7.13 | 7.03 | 9.28 | 9.25 |
| | Yes | 10.20 | 9.61 | 7.17 | 5.26 | 7.16 | 7.32 | 9.47 | 9.40 |
| Avilamycin[2] | No | 10.16 | 9.57 | 7.14 | 5.46 | 7.02 | 7.15 | 9.36 | 9.32 |
| | Yes | 10.16 | 9.56 | 7.11 | 5.18 | 7.28 | 7.20 | 9.38 | 9.33 |
| Treatments [3] | | | | | | | | | |
| CON | | 10.07 | 9.47 | 7.01 | 5.50 | 7.03 | 6.97 | 9.24 | 9.18 |
| OA | | 10.25 | 9.67 | 7.27 | 5.41 | 7.00 | 7.32 | 9.49 | 9.46 |
| OAAV | | 10.14 | 9.55 | 7.07 | 5.11 | 7.32 | 7.32 | 9.45 | 9.34 |
| AV | | 10.17 | 9.56 | 7.15 | 5.24 | 7.23 | 7.08 | 9.31 | 9.32 |
| Statistics | | | | | | | | | |
| SEM[4] | | 0.067 | 0.084 | 0.244 | 0.258 | 0.173 | 0.197 | 0.077 | 0.070 |
| PO | | 0.270 | 0.278 | 0.711 | 0.654 | 0.854 | 0.143 | 0.018 | 0.040 |
| PA | | 0.943 | 0.875 | 0.904 | 0.291 | 0.147 | 0.773 | 0.831 | 0.886 |
| $P_{O \times A}$ | | 0.127 | 0.213 | 0.502 | 0.940 | 0.726 | 0.756 | 0.437 | 0.071 |

[1] No: no OABF addition; Yes: addition of 1 g OABF/kg diet.
[2] No: no avilamycin addition; Yes: addition of 2.5 mg of avilamycin/kg diet.
[3] Interaction means from 8 replicate pens per treatment. Treatments include: control − no additions (CON), 1 g OABF/kg diet (OA), avilamycin 2.5 mg active components/kg diet (AV) and combination of OA + AV (OAAV).
[4] Pooled standard error of means.

decreased the expression of *MUC2* ($P_O = 0.027$), *ZO1* ($P_O = 0.016$) and *CLDN5* ($P_O = 0.003$), whereas avilamycin inclusion significantly decreased the expression levels of *ZO1, CLDN5, OCLN, NFKB1, TLR2B* and *TLR4* ($P_A = 0.016$, $P_A = 0.001$, $P_A = 0.018$, $P_A = 0.024$, $P_A = 0.011$ and $P_A = 0.014$, respectively).

In addition, significant OABF × avilamycin interactions were noted for the expression of *ZO1* ($P_{O \times A} = 0.007$), *ZO2* ($P_{O \times A} = 0.027$), *CLDN1* ($P_{O \times A} = 0.012$), and *CLDN5* ($P_{O \times A} = 0.036$). In particular, broilers in CON showed the highest *ZO1* expression compared with those in the other 3 treatments. In addition, the expression of *ZO2* was higher in CON than in OA and AV, with OAAV being intermediate. Furthermore, *CLDN1* expression was higher in CON than in AV, whereas OAAV and OA were intermediate. Moreover, OAAV, AV and OA had lower *CLDN5* expression than CON.

### 3.4.2. Cecum

From the genes studied, OABF up-regulated the relative expression of *MUC2* ($P_O = 0.014$), whereas, avilamycin addition significantly down-regulated ($P_A = 0.044$) *IgA* (Table 13).

In addition, an OABF × avilamycin interaction was noted for the relative expression of *IgA* ($P_{O \times A} < 0.001$). In particular, the highest IgA expression was noted for broilers of treatment CON and the lowest for broilers of treatment AV with treatments OA and OAAV being intermediate.

## 4. Discussion

It is generally accepted that gut microbiota contributes significantly to the intestinal function and thus has significant impact on

**Table 7**
Effect of dietary inclusion of an organic acids-based formulation (OABF) and avilamycin dietary inclusion on cecal mucosa-associated bacteria (log cells/g mucosa-associated cell pellet) of 42-day-old broilers.

| item | | Total bacteria | *Bacteroides* spp. | *Lactobacillus* spp. | *Clostridium leptum* subgroup | *Clostridium coccoides* subgroup |
|---|---|---|---|---|---|---|
| Main effect | | | | | | |
| OABF[1] | No | 8.56 | 7.98 | 8.02 | 8.02 | 7.76 |
| | Yes | 8.59 | 8.00 | 8.11 | 8.11 | 7.72 |
| Avilamycin[2] | No | 8.52 | 7.94 | 7.96 | 7.96 | 7.65 |
| | Yes | 8.63 | 8.04 | 8.16 | 8.16 | 7.82 |
| Treatments [3] | | | | | | |
| CON | | 8.55 | 7.95 | 7.98 | 7.98 | 7.76 |
| OA | | 8.49 | 7.92 | 7.95 | 7.95 | 7.56 |
| OAAV | | 8.70 | 8.08 | 8.27 | 8.27 | 7.88 |
| AV | | 8.57 | 8.00 | 8.05 | 8.05 | 7.77 |
| Statistics | | | | | | |
| SEM[4] | | 0.158 | 0.169 | 0.229 | 0.161 | 0.148 |
| $P_O$ | | 0.828 | 0.902 | 0.578 | 0.579 | 0.787 |
| $P_A$ | | 0.469 | 0.545 | 0.842 | 0.228 | 0.263 |
| $P_{O \times A}$ | | 0.574 | 0.754 | 0.699 | 0.435 | 0.319 |

[1] No: no OABF addition; Yes: addition of 1 g OABF/kg diet.
[2] No: no avilamycin addition; Yes: addition of 2.5 mg of avilamycin/kg diet.
[3] Interaction means from 8 replicate pens per treatment. Treatments include: control − no additions (CON), 1 g OABF/kg diet (OA), avilamycin 2.5 mg active components/kg diet (AV) and combination of OA + AV (OAAV).
[4] Pooled standard error of means.

**Table 8**
Effect of dietary inclusion of an organic acids-based formulation (OABF) and avilamycin dietary inclusion on microbial glycolytic enzyme activity (μmol p-nitrophenol released/min per g digesta soluble protein) at ileal digesta of 42-day-old broilers.

| Item | | α-glucosidase | β-glucosidase | α-galactosidase | β-galactosidase | β-glucuronidase |
|---|---|---|---|---|---|---|
| Main effect | | | | | | |
| OABF[1] | No | 22.43 | 16.72 | 24.55 | 22.34 | 17.99 |
| | Yes | 26.95 | 21.32 | 33.61 | 30.68 | 24.44 |
| Avilamycin[2] | No | 25.97 | 19.86 | 29.54 | 24.84 | 21.12 |
| | Yes | 23.41 | 18.17 | 28.62 | 28.18 | 21.31 |
| Treatments[3] | | | | | | |
| CON | | 23.41 | 17.08 | 24.64 | 21.24 | 17.42 |
| OA | | 28.53 | 22.64 | 34.45 | 28.43 | 24.81 |
| OAAV | | 25.37 | 20.00 | 32.77 | 32.93 | 24.06 |
| AV | | 21.46 | 16.35 | 24.47 | 23.43 | 18.55 |
| Statistics | | | | | | |
| SEM[4] | | 1.950 | 1.754 | 3.713 | 4.288 | 2.124 |
| $P_O$ | | 0.028 | 0.014 | 0.021 | 0.062 | 0.005 |
| $P_A$ | | 0.201 | 0.344 | 0.806 | 0.442 | 0.929 |
| $P_{O \times A}$ | | 0.758 | 0.590 | 0.841 | 0.789 | 0.663 |

[1] No: no OABF addition; Yes: addition of 1 g OABF/kg diet.
[2] No: no avilamycin addition; Yes: addition of 2.5 mg of avilamycin/kg diet.
[3] Interaction means from 8 replicate pens per treatment. Treatments include: control − no additions (CON), 1 g OABF/kg diet (OA), avilamycin 2.5 mg active components/kg diet (AV) and combination of OA + AV (OAAV).
[4] Pooled standard error of means.

the growth and health of chickens (Gong et al., 2007). The vast majority of gut bacteria resides in the distal intestine, particularly in the ceca, which are mainly colonized by obligate anaerobes (Oakley et al., 2014; Asrore et al., 2015).

In this study, real time PCR was used to determine the effect of dietary inclusion of an OABF and/or avilamycin used as an AGP model for comparison on selected dominant commensal microbiota constituents in broiler ileal and cecal mucosa as well as luminal digesta. In particular, at the ileal level, treatment OAAV resulted in lower total mucosal-associated bacterial levels compared with treatment AV. On the other hand, avilamycin reduced ileal digesta *C. perfringens* counts. Moreover, avilamycin is known to display bactericidal activity against Gram-positive bacteria (La-ongkhum et al., 2011) such as *C. perfringens* (Knarreborg et al., 2002; Van Immerseel et al., 2004) and therefore its inclusion as a positive control in this study could explain the reduced ileal digesta counts of *C. perfringens*. Generally, organic acids have been shown to possess antimicrobial properties (Van Immerseel et al., 2006; Mani-Lopez et al., 2012) that may affect ileal lactic acid bacteria and *E. coli* (Pirgozliev et al., 2008; Nava et al., 2009; Hashemi et al., 2012; Sun et al., 2013) and this could partly explain the reduction in the overall population of ileal mucosa-associated bacteria in this study.

The limited organic acids effects on ileal microbiota composition could be associated with the negligible changes on ileal VFA concentration and profile. However, the activities of microbial glycolytic enzymes determined in ileal digesta were significantly increased by OABF. This fact may imply metabolic stimulation of ileal microbiota, for example of α-glucosidase, β-glucosidase and α-galactosidase, could point to an increased overall digestive capacity for starch, non-starch polysaccharides and dietary α-galactosides (e.g., rafinose and stachyose), respectively (Mountzouris et al., 2007). The aforementioned improved digestive capacity could have had a positive effect on the overall nutrient digestibility, energy salvage and broiler performance reported by Palamidi et al. (2016).

At the cecal level, OABF and/or avilamycin inclusion had no effect on the mucosa-associated microbiota constituents examined. However, cecal digesta *C. coccoides* subgroup and *C. leptum* subgroup levels were significantly increased by dietary OABF inclusion by 0.15 log and 0.19 log, respectively, compared with that in the

**Table 9**
Effect of dietary inclusion of an organic acids-based formulation (OABF) and avilamycin dietary inclusion on microbial glycolytic enzyme activity (μmol p-nitrophenol released/min per g digesta soluble protein) at cecal digesta of 42-day-old broilers.

| Item | | α-glucosidase | β-glucosidase | α-galactosidase | β-galactosidase | β-glucuronidase |
|---|---|---|---|---|---|---|
| Main effect | | | | | | |
| OABF[1] | No | 58.81 | 36.08 | 38.65 | 86.63 | 76.92 |
| | Yes | 59.68 | 35.83 | 40.88 | 66.50 | 78.38 |
| Avilamycin[2] | No | 63.33 | 38.89 | 43.12 | 99.25 | 78.17 |
| | Yes | 55.17 | 33.03 | 36.41 | 53.89 | 77.13 |
| Treatments[3] | | | | | | |
| CON | | 56.98 | 34.64 | 36.88 | 112.00 | 75.36 |
| OA | | 69.67 | 43.13 | 49.37 | 86.49 | 80.97 |
| OAAV | | 49.68 | 28.53 | 32.40 | 46.51 | 75.78 |
| AV | | 60.65 | 37.53 | 40.41 | 61.26 | 78.48 |
| Statistics | | | | | | |
| SEM[4] | | 6.700 | 5.248 | 5.352 | 19.913 | 11.878 |
| $P_O$ | | 0.899 | 0.962 | 0.679 | 0.321 | 0.903 |
| $P_A$ | | 0.233 | 0.274 | 0.220 | 0.031 | 0.931 |
| $P_{O \times A}$ | | 0.088 | 0.107 | 0.066 | 0.789 | 0.729 |

[1] No: no OABF addition; Yes: addition of 1 g OABF/kg diet.
[2] No: no avilamycin addition; Yes: addition of 2.5 mg of avilamycin/kg diet.
[3] Interaction means from 8 replicate pens per treatment. Treatments include: control − no additions (CON), 1 g OABF/kg diet (OA), avilamycin 2.5 mg active components/kg diet (AV) and combination of OA + AV (OAAV).
[4] Pooled standard error of means.

**Table 10**
Effect of dietary inclusion of an organic acids-based formulation (OABF) and avilamycin dietary inclusion on the volatile fatty acid (VFA) concentration and molar ratios in the ileal digesta of 42-day-old broilers.

| Item | | Total VFA[1], mmol/kg of wet ileal digesta | Acetic, % | Propionic, % | Butyric, % | Other VFA[2], % | Branched VFA[3], % |
|---|---|---|---|---|---|---|---|
| Main effect | | | | | | | |
| OABF[4] | No | 8.17 | 69.88 | 3.91 | 15.23 | 6.38 | 4.62 |
| | Yes | 9.05 | 73.65 | 3.62 | 12.46 | 5.59 | 4.68 |
| Avilamycin[5] | No | 8.63 | 75.63 | 3.49 | 11.17 | 5.74 | 3.98 |
| | Yes | 8.59 | 67.91 | 4.04 | 16.52 | 6.23 | 5.32 |
| Treatments[6] | | | | | | | |
| CON | | 7.79 | 73.61 | 3.60 | 12.34 | 6.51 | 3.94 |
| OA | | 9.48 | 77.64 | 3.38 | 10.00 | 4.96 | 4.03 |
| OAAV | | 8.63 | 69.66 | 3.86 | 14.93 | 6.23 | 5.34 |
| AV | | 8.55 | 66.15 | 4.21 | 18.11 | 6.24 | 5.30 |
| Statistics | | | | | | | |
| SEM[7] | | 0.800 | 3.160 | 0.511 | 1.940 | 0.714 | 0.731 |
| $P_O$ | | 0.280 | 0.243 | 0.578 | 0.166 | 0.283 | 0.932 |
| $P_A$ | | 0.957 | 0.021 | 0.291 | 0.010 | 0.495 | 0.078 |
| $P_{O \times A}$ | | 0.322 | 0.936 | 0.904 | 0.828 | 0.291 | 0.973 |

[1] Total VFA = acetic + propionic + butyric + branched VFA + other VFA.
[2] Other VFA = valeric + caproic + heptanoic.
[3] Branched VFA = isobutyric + isovaleric + isocaproic.
[4] No: no OABF addition; Yes: addition of 1 g OABF/kg diet.
[5] No: no avilamycin addition; Yes: addition of 2.5 mg of avilamycin/kg diet.
[6] Interaction means from 8 replicate pens per treatment. Treatments include: control – no additions (CON), 1 g OABF/kg diet (OA), avilamycin 2.5 mg active components/kg diet (AV) and combination of OA + AV (OAAV).
[7] Pooled standard error of means.

non-OABF supplemented birds. Effects of organic acids on cecal microbiota members such as total bacteria and *Salmonella* have been also reported by other studies (Hamed and Hassan, 2013; Fernandez-Rubio et al., 2009). The noted increases in *C. coccoides* subgroup and *C. leptum* subgroup, also known as *Clostridium* XIVa and *Clostridium* IV clusters, although were low, they could be regarded as important and beneficial for gut health. These clusters are among the most abundant cecal digesta bacteria (Gong et al., 2007) including a large number of butyrate producers that are valuable for gut homeostasis (Lopetuso et al., 2013).

Unlike in the ileum, the changes in cecal microbiota composition were not associated with significant changes in microbial metabolic activity. This could in part be attributed to the highly digestible diets used in this study, which is in contrast with other studies where the presence of non-digestible carbohydrates in broiler ceca yielded differences in VFA profile and concentration (Jozefiak et al., 2004; Kiarie et al., 2014). As it was expected, the total VFA concentration was lower in the ileum than in the ceca since bacterial fermentation is limited in the small intestine of broilers due to the short digesta transit time (Rehman et al., 2007).

In the present study, OABF inclusion resulted in downregulation of expression of genes encoding tight junction proteins (*ZO1, CLDN5*) and *MUC2* in the ileal mucosa. Furthermore, avilamycin inclusion used as a positive control in this study also downregulated the expression of tight junction proteins (*ZO1, CLDN5, OCLN*). Reduced gene expression of tight junction proteins and

**Table 11**
Effect of dietary inclusion of an organic acids-based formulation (OABF) and avilamycin dietary inclusion on the volatile fatty acid (VFA) concentration and molar ratios in the cecal digesta of 42-day-old broilers.

| Item | | Total VFA[1], mmol/kg of wet cecal digesta | Acetic, % | Propionic, % | Butyric, % | Other VFA[2], % | Branched VFA[3], % |
|---|---|---|---|---|---|---|---|
| Main effect | | | | | | | |
| OABF[4] | No | 112.44 | 62.23 | 9.28 | 23.50 | 2.04 | 2.94 |
| | Yes | 102.36 | 62.25 | 9.08 | 23.13 | 2.32 | 3.21 |
| Avilamycin[5] | No | 112.41 | 62.04 | 9.73 | 23.08 | 2.04 | 3.09 |
| | Yes | 102.39 | 62.44 | 8.64 | 23.54 | 2.33 | 3.06 |
| Treatments[6] | | | | | | | |
| CON | | 110.80 | 63.03 | 9.09 | 22.66 | 2.03 | 3.19 |
| OA | | 114.01 | 61.06 | 10.36 | 23.50 | 2.05 | 3.00 |
| OAAV | | 90.70 | 63.44 | 7.80 | 22.75 | 2.59 | 3.43 |
| AV | | 114.09 | 61.44 | 9.48 | 24.34 | 2.06 | 2.70 |
| Statistics | | | | | | | |
| SEM[7] | | 9.467 | 1.247 | 0.783 | 1.870 | 0.136 | 0.406 |
| $P_O$ | | 0.296 | 0.988 | 0.800 | 0.843 | 0.052 | 0.513 |
| $P_A$ | | 0.299 | 0.755 | 0.176 | 0.806 | 0.043 | 0.939 |
| $P_{O \times A}$ | | 0.171 | 0.123 | 0.070 | 0.522 | 0.076 | 0.270 |

[1] Total VFA = acetic + propionic + butyric + branched VFA + other VFA.
[2] Other VFA = valeric + caproic + heptanoic.
[3] Branched VFA = isobutyric + isovaleric + isocaproic.
[4] No: no OABF addition; Yes: addition of 1 g OABF/kg diet.
[5] No: no avilamycin addition; Yes: addition of 2.5 mg of avilamycin/kg diet.
[6] Interaction means from 8 replicate pens per treatment. Treatments include: control – no additions (CON), 1 g OABF/kg diet (OA), avilamycin 2.5 mg active components/kg diet (AV) and combination of OA + AV (OAAV).
[7] Pooled standard error of means.

**Table 12**
Effect of dietary inclusion of an organic acids-based formulation (OABF) and avilamycin dietary inclusion on relative mRNA levels of ileal mucosa barrier genes of 42-day-old broilers.

| Item | | *MUC2* | *IgA* | *ZO1* | *ZO2* | *CLDN1* | *CLDN5* | *OCLN* | *NFKB1* | *TLR2B* | *TLR4* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Main effect | | | | | | | | | | | |
| OABF[1] | No | 1.196 | 2.925 | 1.89 | 1.52 | 1.32 | 1.79 | 1.52 | 1.23 | 1.97 | 2.07 |
| | Yes | 0.851 | 3.426 | 1.00 | 1.10 | 1.38 | 0.83 | 0.99 | 1.24 | 1.47 | 1.16 |
| Avilamycin[2] | No | 1.019 | 3.412 | 1.88 | 1.42 | 1.64 | 1.86 | 1.60 | 1.55 | 2.49 | 2.25 |
| | Yes | 1.028 | 2.938 | 1.01 | 1.20 | 1.05 | 0.77 | 0.91 | 0.92 | 0.96 | 0.97 |
| Treatments[3] | | | | | | | | | | | |
| CON | | 1.049 | 3.753 | 2.82$^{a}$ | 2.03$^{a}$ | 2.06$^{a}$ | 2.67$^{a}$ | 2.14 | 1.74 | 3.06 | 3.11 |
| OA | | 0.988 | 3.072 | 0.94$^{b}$ | 0.81$^{b}$ | 1.23$^{ab}$ | 1.04$^{b}$ | 1.07 | 1.35 | 1.92 | 1.39 |
| OAAV | | 0.714 | 3.780 | 1.07$^{b}$ | 1.40$^{ab}$ | 1.53$^{ab}$ | 0.62$^{b}$ | 0.91 | 1.12 | 1.02 | 0.92 |
| AV | | 1.343 | 2.096 | 0.95$^{b}$ | 1.02$^{b}$ | 0.57$^{b}$ | 0.92$^{b}$ | 0.90 | 0.72 | 0.89 | 1.02 |
| Statistics | | | | | | | | | | | |
| SEM[4] | | 0.148 | 0.770 | 0.344 | 0.341 | 0.334 | 0.331 | 0.295 | 0.262 | 0.565 | 0.490 |
| $P_O$ | | 0.027 | 0.520 | 0.016 | 0.228 | 0.859 | 0.003 | 0.169 | 0.974 | 0.381 | 0.073 |
| $P_A$ | | 0.947 | 0.543 | 0.016 | 0.533 | 0.085 | 0.001 | 0.018 | 0.024 | 0.011 | 0.014 |
| $P_{O \times A}$ | | 0.065 | 0.136 | 0.007 | 0.027 | 0.012 | 0.036 | 0.125 | 0.140 | 0.272 | 0.110 |

*MUC2* = mucin 2; *IgA* = immunoglobulin A; *ZO1* = zonula occludens-1; *ZO2* = zonula occludens-2; *CLDN1* = claudin-1; *CLDN5* = claudin-5; *CLDN* = occludin; *NFKB1* = nuclear factor kappa B subunit 1; *TLR2B* = toll-like receptor 2 family member B; *TLR4* = toll-like receptor 4.
[a, b] Interaction means within the same column differ significantly ($P \leq 0.05$).
[1] No: no OABF addition; Yes: addition of 1 g OABF/kg diet.
[2] No: no avilamycin addition; Yes: addition of 2.5 mg of avilamycin/kg diet.
[3] Interaction means from 8 replicate pens per treatment. Treatments include: control − no additions (CON), 1 g OABF/kg diet (OA), avilamycin 2.5 mg active components/kg diet (AV) and combination of OA + AV (OAAV).
[4] Pooled standard error of means.

*MUC2* is usually associated with pathogenic challenge and pathological conditions (Cox et al., 2010; Zhang et al., 2012; Wang et al., 2014; Antonissen et al., 2015; Chen et al., 2015; Lee et al., 2017) that are characterized by severe ileal inflammation (Antonissen et al., 2015; Chen et al., 2015). Generally, the gut is an organ that remains under a physiological state of mild inflammation when exposed to an array of continuous challenges (O'Hara and Shanahan, 2006; O'Flaherty et al., 2010). However, in this study no sign of abnormal inflammation was present in any of the treatments. The latter could be also supported by the unaffected ileal mucosa IgA levels. Moreover, the fact that the birds from this study had improved zootechnical performance and nutrient digestibility (Palamidi et al., 2016) could provide further proof for the absence of abnormal inflammation. The downregulated expression of tight junction proteins in AV group could be explained by considering the postulated anti-inflammatory role of avilamycin and other AGP (Costa et al., 2011; Niewold, 2007) in the absence of pathogenic challenges as in this study.

Defense against pathogens and maintenance of homeostasis are dependent on signaling pathways induced by receptors such as toll-like receptors (TLRs). Toll-like receptors sense the presence of conserved microbial structures in the environment and instruct the eukaryotic cells to an adequate response; TLR2 and TLR4 recognize mainly bacterial cell wall components of Gram-positive and Gram-negative bacteria, respectively (St Paul et al., 2013). A major signaling target of the TLRs is activation of the transcription factor

**Table 13**
Effect of dietary inclusion of an organic acids-based formulation (OABF) and avilamycin dietary inclusion on relative mRNA levels of cecal mucosa barrier genes of 42-day-old broilers.

| Item | | *MUC2* | *IgA* | *ZO1* | *ZO2* | *CLDN1* | *CLDN5* | *OCLN* | *NFKB1* | *TLR2B* | *TLR4* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Main effect | | | | | | | | | | | |
| OABF[1] | No | 0.752 | 1.069 | 1.24 | 1.20 | 1.29 | 1.07 | 1.29 | 1.11 | 1.17 | 1.03 |
| | Yes | 1.136 | 0.933 | 1.32 | 1.05 | 1.22 | 1.31 | 1.27 | 1.48 | 1.46 | 1.48 |
| Avilamycin[2] | No | 0.974 | 1.154 | 1.11 | 1.09 | 1.23 | 1.07 | 1.02 | 1.28 | 1.29 | 1.11 |
| | Yes | 0.914 | 0.848 | 1.45 | 1.16 | 1.27 | 1.30 | 1.53 | 1.31 | 1.35 | 1.40 |
| Treatments[3] | | | | | | | | | | | |
| CON | | 0.696 | 1.530$^{a}$ | 1.12 | 1.32 | 1.32 | 0.86 | 1.27 | 1.34 | 1.00 | 0.72 |
| OA | | 1.251 | 0.778$^{bc}$ | 1.10 | 0.85 | 1.14 | 1.29 | 0.79 | 1.21 | 1.58 | 1.49 |
| OAAV | | 1.021 | 1.089$^{b}$ | 1.55 | 1.24 | 1.29 | 1.33 | 1.74 | 1.74 | 1.34 | 1.47 |
| AV | | 0.807 | 0.608$^{c}$ | 1.35 | 1.07 | 1.26 | 1.27 | 1.31 | 0.87 | 1.35 | 1.33 |
| Statistics | | | | | | | | | | | |
| SEM[4] | | 0.146 | 0.145 | 0.338 | 0.222 | 0.319 | 0.279 | 0.378 | 0.322 | 0.346 | 0.309 |
| $P_O$ | | 0.014 | 0.358 | 0.795 | 0.509 | 0.813 | 0.391 | 0.852 | 0.259 | 0.411 | 0.157 |
| $P_A$ | | 0.685 | 0.044 | 0.327 | 0.751 | 0.883 | 0.417 | 0.154 | 0.931 | 0.866 | 0.350 |
| $P_{O \times A}$ | | 0.252 | <0.001 | 0.736 | 0.161 | 0.745 | 0.522 | 0.211 | 0.130 | 0.401 | 0.323 |

*MUC2* = mucin 2; *IgA* = immunoglobulin A; *ZO1* = zonula occludens-1; *ZO2* = zonula occludens-2; *CLDN1* = claudin-1; *CLDN5* = claudin-5; *CLDN* = occludin; *NFKB1* = nuclear factor kappa B subunit 1; *TLR2B* = toll-like receptor 2 family member B; *TLR4* = toll-like receptor 4.
[a–c] Interaction means within the same column differ significantly ($P \leq 0.05$).
[1] No: no OABF addition; Yes: addition of 1 g OABF/kg diet.
[2] No: no avilamycin addition; Yes: addition of 2.5 mg of avilamycin/kg diet.
[3] Interaction means from 8 replicate pens per treatment. Treatments include: control − no additions (CON), 1 g OABF/kg diet (OA), avilamycin 2.5 mg active components/kg diet (AV) and combination of OA + AV (OAAV).
[4] Pooled standard error of means.

NF-κB, a key regulator of immune and inflammatory responses (Zhang and Ghosh, 2001). Organic acids-based formulation inclusion did not affect ileal mucosa *TLR2*, *TLR4* and *NF-κB* expression. A possible explanation for this could be that the OABF inclusion did not also affect the ileal microbiota. Indeed, it is known that most commensal bacteria do not activate or limit NF-kB signaling and that in a healthy gut *TLR* expression profiles remain low and contribute to gut homeostasis (O'Hara and Shanahan, 2006; Cario, 2010). On the other hand, avilamycin inclusion reduced ileal mucosa *TLR2b*, *TLR4* and *NFKB1* expression. This could be explained by the avilamycin induced reduction of *C. perfringens* subgroup levels shown earlier, and/or to an avilamycin anti-inflammatory effect (Costa et al., 2011; Niewold, 2007), through a bacteria-independent inhibition of TLRs.

At cecal level *IgA* expression was downregulated in treatments OA, OAAV and AV compared with the control. To the best of our knowledge there is no other scientific publication dealing with OABF effects on cecal *IgA* expression. However, it could be the result of an overall better management of the cecal environment. On the other hand, cecal *MUC2* was upregulated by OABF addition. It is known that intestinal microbiota can affect mucin turnover by stimulation of mucin gene expression (Smirnov et al., 2005). Therefore, the observed upregulation of *MUC2* expression could be linked with the increases in the levels of *C. leptum* and *C. coccoides* subgroups.

## 5. Conclusions

This study has provided additional evidence that diet supplementation with OABF can positively affect cecal microbiota composition and activity of ileal microbial glycolytic enzymes. The expression of genes associated with gut barrier and health was shown to be mostly modulated in the ileum rather than in the ceca. Synergies of OABF with avilamycin were shown for ileal tight junction proteins and cecal *IgA* gene expression. All the above point to an OABF potential to manipulate the intestinal environment that should however be further assessed under stress challenge conditions.

## Conflicts of interest

The authors declare that they have no conflict of interest.

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