Effect of Dietary Organic Acids and Humic Substance Supplementation on Performance, Immune Response and Gut Morphology of Broiler Chickens

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Primary Audience: Nutrition

SUMMARY

This study evaluated the additive effects of a commercial feed supplementation blend (Ava Cid P)—consisting of humic substances, coated sodium butyrate, and a small acidifier portion—on the growth, immune response, and gut health of broiler chickens. A total of 540 female and 540 male broilers were raised from 1–49 d. On the first day, the animals were distributed in a completely randomized 2 × 5 factorial design (2 sexes and 5 treatments) with 7 replications of 15 birds each. The 5 treatments were 1) birds did not receive Ava Cid P (control); 2) birds received 0.91 kg/t of Ava Cid P from 1–21 d (AVA1–21); 3) birds received 0.91 kg/t of Ava Cid P from 1–21 d and 0.45 kg/t from 22–35 d (AVA1–35); 4) birds received 0.91 kg/t of Ava Cid P from 1–21 d and 0.45 kg/t from 22–42 d (AVA1–42); and 5) birds received 0.91 kg/t of Ava Cid P from 1–21 d, 0.45 kg/t from 22–35 d, and 0.23 kg/t from 36–49 d (AVA1–49). ANOVA and Tukey's tests were applied to compare the means ($P < 0.05$) between treatments. The Ava Cid P showed no effect on male or female growth performance or goblet cell density. However, the supplement modified gut morphometry, and jejunum villi were 32% higher at 9 and 35 d in the AVA1–35 birds compared with those of the control group. The apparent villus surface and villus height increased by 87% and 46%, respectively, in the AVA1–49 birds compared with the AVA1–21 birds. The expression of mucin 2 (MUC2) and tumor necrosis factor-alpha (TNF-α) were 1.6% and 0.9% lower in the AVA1–21 birds than in the control birds, but no effects were observed for interleukin-1 beta and interleukin-10. The Ava Cid P altered the mRNA expression of MUC2 and TNF-α and some characteristics of intestinal morphometry, but did not change the performance of broilers.

Key words: animal performance, sodium butyrate, gut morphometry, humic acid

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DESCRIPTION OF PROBLEM

Growing concern about the transmission and proliferation of resistant bacteria via food chains has led to a ban on antibiotic growth promoters (AGP) in livestock in the European Union since 2006 [1]. Despite international regulations, modern intensive farming, high densities, and high yield requirements render commercial broiler chickens vulnerable to different stressors, including intestinal and immunological stress; these could be controlled before, and in part, by AGP. Coupled with these facts, there is increasing pressure to prohibit the use of AGP based on the possibility of allergic reactions and cross-resistance of pathogenic bacterial strains in humans. These facts have been forcing the countries that export animal products to search for alternatives to ensure maximum animal growth without affecting the quality of the final product. In this context, organic acids are one possibility for non-AGP.

In the animal feed industry, organic acids are added to reduce the action of bacteria such as *Escherichia coli*, *Campylobacter* spp., and *Salmonella* spp. in contaminated feed [2], thereby reducing subclinical infections in birds. Organic acids may be exploited as growth promoters in broiler chickens due to their beneficial antimicrobial effects and positive effects on the histology of the small intestine. The organic acids improve villus height in the small intestine and have a direct stimulatory effect on gastrointestinal cell proliferation, thereby facilitating nutrient absorption and growth performance [3].

Butyric acid is a natural substance present in the gut, milk, and feces of most mammals. It is available as Na, K, Mg, or Ca salt. In vitro and in vivo studies have shown that butyric acid, or its sodium salt, can decrease pro-inflammatory cytokine expression and release [4–6]. Zhang et al. [5] showed inhibition of serum IL-6 and TNF elevation in LPS-challenged chickens but decreases in those concentrations in normally reared broilers. Moreover, butyric acid and its sodium salt have received attention as feed additives, and some studies have demonstrated positive effects on the growth performance of chickens, such as decreased tissue catabolism, intestinal integrity, and trophic effects on the gastrointestinal tract in broilers [5–8].

Humic substances or humates, such as reed sedge peat compounds, are composed of humic acid, fulvic acid, and trace minerals. These substances have been shown to transfer micronutrients from soil to plants, enhance water retention, and improve microbial populations in soils [9]. Humate supplementation increased the feed conversion efficiency and egg production in hens, but had no effect on feed intake [10]. Humate supplementation does not improve growth by affecting feed intake per se; the improvement in weight gain and better feed conversion may be related to promotional effects on metabolic processes of digestion and utilization of nutrients [11]. Humates have also been associated with improved health through physiological changes and development of immunity in different animal species [12].

Ava Cid P is a commercial blend composed of a reed sedge peat compound, coated sodium butyrate 30%, and a small acidifier component containing phosphoric, citric, malic, and fumaric acids. The purpose of this study was to evaluate the effects of the combination of these three substances and dose responses of Ava Cid P on the performance, immune response, and gut health of broiler chickens in each phase of growth.

MATERIALS AND METHODS

The experiment was conducted at the Piedmont Research Station, North Carolina Department of Agriculture & Consumer Services, NC Station, Salisbury, NC, USA. All procedures were approved by the Animal Care and Welfare Committee of NC State University. A total of 1,080 one-day-old (540 females, 540 males) Ross 344 × 780 broiler chicks, separated by sex, were used. The birds' average weight was 42.50 g ± 5.0%. The broilers were housed in a building with floor pens equipped with nipple drinkers and feeders. Environmental temperatures were managed with heaters to maintain birds in thermoneutral conditions during the experimental period.

The experiment was divided into three phases: starter (1 to 21 d), growth (22 to 35 d), and final (36 to 49 d). Birds were fed with a pelleted diet in all phases (Table 1), and water and feed were provided *ad libitum*. The

Table 1. Ingredients and Nutrients of Experimental Basal Diets as Fed Basis.

Item	Broiler starter (1–21 d)	Broiler grower (21–35 d)	Broiler finisher (35–49 d)
Ingredients (%)			
Corn	57.16	63.43	71.94
Soybean meal, 48% CP	38.31	31.51	23.18
Vegetal oil[1]	1.40	1.91	1.92
Dicalcium phosphate	1.69	1.74	1.52
Limestone	0.67	0.58	0.58
Salt	0.45	0.45	0.45
L-Lys HCl	0.01	0.03	0.10
DL-Met	0.22	0.17	0.12
L-Tre	0.01	0.00	0.00
Mineral premix[2]	0.10	0.10	0.10
Vitaminic premix[2]	0.05	0.05	0.05
Nutricional values			
ME, kcal/kg	2950	3050	3150
Crude protein, %	23.18	20.30	17.00
Crude fat, %	2.99	4.79	5.58
Crude fibre, %	2.32	2.57	2.49
Calcium, %	0.90	0.90	0.80
Total phosphorus, %	0.719	0.697	0.626
Avail. Phos.	0.450	0.450	0.400
Sodium, %	0.200	0.200	0.200
Potassium, %	1.122	0.934	0.743
Chloride, %	0.317	0.332	0.348
Na+k-cl, meq/kg	284.96	232.48	179.11
Arginine, %	1.5582	1.2970	1.0381
Dig. Arg., %	1.4354	1.1907	0.9529
Lysine, %	1.3000	1.1400	0.9700
Dig. Lys, %	1.1625	1.0179	0.8718
Methionine, %	0.5741	0.4921	0.4146
Dig. Met chicken, %	0.5354	0.4536	0.3798
Met + cys, %	0.9500	0.8320	0.7081
Dig. Tsaa, %	0.7867	0.6877	0.5888
Threonine, %	0.8892	0.7638	0.6500
Dig. Thr, %	0.7835	0.6647	0.5649
Tryptophan, %	0.2649	0.2182	0.1705
Dig. Trp, %	0.2318	0.1899	0.1476
Leucine, %	1.9375	1.7685	1.5292
Isoleucine, %	0.9856	0.8419	0.6812
Valine, %	1.1813	1.0250	0.8399

experimental design was completely randomized in a 2 × 5 factorial arrangement (2 sex × 5 treatments), with 7 replicates of 15 birds each.

The experimental diets were composed of different levels and periods of supplementation as follows: 1) birds did not receive Ava Cid P in any phase (Control); 2) birds received 0.91 kg/t of Ava Cid P from 1 to 21 d ($AVA_{1–21}$); 3) birds received 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22 to 35 d ($AVA_{1–35}$); 4) birds received 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22 to 42 d ($AVA_{1–42}$); and 5) birds received 0.91 kg/t of Ava Cid P from 1 to 21 d, 0.45 kg/t from 22 to 35 d, and 0.23 kg/t from 36 to 49 d ($AVA_{1–49}$). All the basal corn-soybean meal diets had the same nutrient levels and no addition of coccidiostats, AGP, or any type of enzyme (Table 1).

Body weight (BW), feed intake (FI), and feed conversion rate were evaluated at 1, 21, 35, 42, and 49 d. Mortality was not assessed in the study. The mortality rate was less than 1% and the performance variables were adjusted according to the weight of the animals.

Histological Analysis

At 9, 35, and 49 d, jejunum and ileum tissue samples were collected from 5 male birds/treatment for intestinal morphometric analysis. The tissue samples were immediately rinsed with saline and fixed in 10% neutral-buffered formalin solution for at least 72 h before processing. A total of 3 sections of approximately 2–3 mm length were taken from a 3 cm fixed jejunum and ileum section collected from each sampled bird. These smaller sections were placed in tissue cassettes and submerged in 10% buffered formalin solution until processed at the Histopathology Laboratory (Pathology Laboratory, NC State University, College of Veterinary Medicine, Raleigh, NC, USA). The fixed sections were embedded in paraffin wax, and 5 μm thick transverse sections were cut with a microtome. The 5 μm cut sections were placed on slides and stained with Lilee Meyer hematoxylin and counter-stained with eosin yellow. A light microscope was used to visualize the transverse sections placed on slides. The images were analyzed using image tool software (Software AmScope 4.7, Irvine, CA, USA). Villus height, villus apical width at the tip of the villus, villus basal width at the crypt-villus junction, crypt depth, and muscularis depth were measured for 10 villi per sampled bird. The villi height: crypt depth ratio for each bird was calculated by dividing the average of the 10 villi heights measured per bird by the average of the 10 crypt depths measured on the same bird. The following mathematical formula was used to determine apparent villus surface area according to Iji et al. [13]: {[(villus tip + villus base)/2] × villus height}. The goblet cell density was calculated from the jejunum and ileum of 5 male birds/treatment at 49 d, by counting the number of Alcian Blue-positive cells along a 200 μm linear area/villus (10 villi/bird).

MUC2 and Cytokine Expression

The quantification of mRNA expression of cytokines interleukin-1 beta (IL-1β), interleukin-10 (IL-10), TNF-α, and MUC2 (by RT-qPCR) was done at 21, 35, and 49 d, on the jejunum of 5 male birds/treatment. Total RNA was isolated (RNeasy®, Mini Kit from Qiagen, Cat. no. 74106) according to the manufacturer's guidelines. Concentration and purity were determined at 260/280 and 260/230 using a spectrophotometer (Spectrophotometer NanoDrop 2000, Thermo Scientific, Wilmington, DE). Reverse transcription was performed with the Kit cDNA (Kit cDNA high Capacity cDNA Reverse Transcription, Applied Biosystems) according to the manufacturer's guidelines.

Relative quantification in real time polymerase chain reaction (PCR) was performed using the Power SYBR® green Master Mix (Power SYBR green Master Mix Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's guidelines. Cycling was carried out in the StepOnePlus™ Real-Time PCR System (StepOnePlus™ Real-Time PCR System, Applied Biosystems). IL-1 primer sequences were 5′-GCTCTACATGTCGTGTGTGATGAG-3′ (forward) and 5′-TGTCGATGTCCCGCATGA-3′ (reverse) [14], IL-10 primer sequences were 5′-CATGCTGCTGGGCCTGAA-3′ (forward) and 5′-CGTCTCCTTGATCTGCTTGATG-3′ (reverse)[14], TNF-α primer sequences were 5′-GAGCGTTGACTTGGCTGTC-3′ (forward) and 5′-AAGCAACAACCAGCTATGCAC-3′ (reverse) [15], MUC2 primer sequences were 5′-AAGCCAGTCTCCTTCAGTAA-3′ (forward) and 5′-TGGTGTGGGAGCAGTGGTT-3′ (reverse), and GAPDH primer sequences were 5′-GGTAAAGTCGGAGTCAACGG-3′ (forward) and 5′-TCGATGAAGGGATCATTGATC-3′ (reverse) (Beacon Designer). Relative mRNA abundance was determined using the 2-ΔΔCt [16] method; Ct values of each sample were standardized for GAPDH RNA.

Statistical Analysis

Data were subject to analysis of variance (ANOVA) using the GLM procedure of JMP (Version 12.0.1, SAS Inst. Inc., 2015), considering dietary inclusion of Ava Cid P and sex as the main factors. Tukey's comparison of means test was applied when significant differences occurred at the 0.05 level of significance.

RESULTS AND DISCUSSION

Supplementation with Ava Cid P showed no effect ($P > 0.05$) on the performance of male

Table 2. Body Weight, Feed Intake and Feed Conversion Ratio of Males and Females in the Experimental Period.

	Male			Female		
Age: 21	BW(g)	FI(g)	FCR	BW(g)	FI(g)	FCR
Control[1]	720	1016	1.418	571	862	1.433
$AVA_{1–21}$	694	979	1.398	596	840	1.412
SEM[2]	0.01	0.01	0.01	0.01	0.01	0.01
P value	0.2969	0.1116	0.5378	0.3679	0.4645	0.4770
Age: 35	BW(g)	FI(g)	FCR	BW(g)	FI(g)	FCR
Control	2007	3010	1.570	1633	2561	1.640
$AVA_{1–21}$	1916	2927	1.610	1646	2483	1.590
$AVA_{1–35}$	1956	2928	1.570	1633	2534	1.630
SEM	0.03	0.04	0.02	0.05	0.04	0.05
P value	0.3019	0.3918	0.0940	0.9533	0.4428	0.5809
Age: 42	BW(g)	FI(g)	FCR	BW(g)	FI(g)	FCR
Control	2628	4211	1.760	2188	3602	1.720
$AVA_{1–21}$	2560	4116	1.740	2169	3497	1.670
$AVA_{1–35}$	2604	4103	1.710	2198	3592	1.720
$AVA_{1–42}$	2548	4087	1.760	2113	3544	1.730
$AVA_{1–49}$	2681	4265	1.730	2185	3562	1.700
SEM	0.03	0.03	0.01	0.02	0.02	0.01
P value	0.4672	0.3646	0.5588	0.6176	0.7325	0.7661
Age: 49	BW(g)	FI(g)	FCR	BW(g)	FI(g)	FCR
Control	3391	5637	1.900	2792	4832	1.800
$AVA_{1–21}$	3347	5497	1.870	2764	4682	1.740
$AVA_{1–35}$	3357	5500	1.840	2865	4815	1.800
$AVA_{1–42}$	3365	5474	1.860	2731	4689	1.780
$AVA_{1–49}$	3475	5732	1.860	2867	4820	1.750
SEM	0.03	0.05	0.02	0.03	0.03	0.02
P value	0.6632	0.3036	0.7958	0.3832	0.4678	0.7128

[1]Control: basal diet without supplementation; $AVA_{1–21}$: 0.91 kg/t of Ava Cid P from 1 to 21 d; $AVA_{1–35}$: 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22 to 35 d; $AVA_{1–42}$: 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22 to 42 d; $AVA_{1–49}$: 0.91 kg/t of Ava Cid P from 1 to 21 d, 0.45 kg/t from 22 to 35 d and 0.23 kg/t from 36 to 49 d.
[2]Pooled SEM (standard error of the means).

and female birds (Table 2) in any growth phase. Zhang et al. [5] also demonstrated no effect of dietary sodium butyrate on broiler growth performance. However, when these same birds were challenged with *Escherichia coli* lipopolysaccharide (LPS), dietary sodium butyrate prevented a reduction in BW gain and feed intake. Edmonds et al. [17] found that during heat stress, broilers fed a mixture of humic acid and protected butyric acid showed improved growth and feed efficiency, along with lower mortality. Our results on performance may be attributable to the environment of the chickens: they were reared inside an experimental house with air conditioning and without any environmental, health, or nutritional stress. The results of Leeson et al. [18] also suggested that there is no growth promoting response to butyric acid or its sodium salt when chickens are reared in an environment with low pathogens or where the health status of the birds is good.

In most cases where benefits of dietary butyric acid, humic substances, or organic acid supplementation on broiler performance were found, researchers used a higher inclusion of feed [17, 19–22]. For example, Antongiovanni et al. [19] used up to 1.0% butyric acid dietary inclusion. Hu and Guo [8] used a similar level of supplementation, but just sodium butyrate, and reported a positive effect on BW gain from 0 to 21 d, which was not maintained up to 42 d. In addition, the results of Biggs and Parsons [23] indicated that feeding 1% to 6% organic acids (citric, gluconic, fumaric, and malic acids), had no consistent effects on growth performance, ME_n, amino acid digestibility, or cecal microbial

Table 3. Goblet Cell Density at 49 d.

Treatment	Jejunum	Ileum
Control[1]	531.80	902.80
$AVA_{1–21}$	519.60	786.00
$AVA_{1–35}$	478.80	776.80
$AVA_{1–42}$	461.40	914.80
$AVA_{1–49}$	578.25	871.20
SEM[2]	21.78	57.81
P value	0.5348	0.9242

[1]Control: basal diet without supplementation; $AVA_{1–21}$: 0.91 kg/t of Ava Cid P from 1 to 21 d; $AVA_{1–35}$: 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22 to 35 d; $AVA_{1–42}$: 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22 to 42 d; $AVA_{1–49}$: 0.91 kg/t of Ava Cid P from 1 to 21 d, 0.45 kg/t from 22 to 35 d and 0.23 kg/t from 36 to 49 d.
[2]Pooled SEM (standard error of the means).

numbers. The lack of positive effects on broiler performance may be associated with the doses of the compounds used in the product. The current formulation of the product, even with the three substances used together, was not able to improve performance.

The histological analysis showed no differences in goblet cell density (Table 3), which was unexpected. Previous studies have shown increased goblet cell counts in unchallenged birds with the use of alternative growth promoters, including butyrate [24, 25] and decreased counts in *Salmonella*-challenged birds fed clays [24]. The differentiation of stem cells in the crypts into goblet cells is increased by inflammation and by stimulation of Krüppel-like factor 4 (KLF4); butyrate can also induce expression of KLF4 [26].

Morphometry was affected by Ava Cid P inclusion (Tables 4 and 5). The jejunum villi height was higher in birds with Ava Cid P inclusion than

Table 4. Effect of the Treatments on the Jejunum Morphometric Analysis.

Age: 9d	Villi height (μm)	Upper villi width (μm)	Botton villi width (μm)	Crypth deep (μm)	Muscularis thickness (μm)	Ratio	Surface of villi (μm^2)
Control[1]	541.67[b]	134.00	174.00	102.67	104.50	5.47	83,321
$AVA_{1–21}$	650.42[a]	138.92	165.71	111.71	93.33	5.92	99,199
SEM[2]	16.83	5.43	7.57	3.87	2.95	0.17	4936
P value	0.0400	0.7820	0.7380	0.4740	0.2111	0.4160	0.3220
Age: 35d	Villi height (μm)	Upper villi width (μm)	Botton villi width (μm)	Crypth deep (μm)	Muscularis thickness (μm)	Ratio	Surface of villi (μm^2)
Control	1061.00[b]	153.67	169.00	124.00	144.33	9.18	173,733
$AVA_{1–21}$	1101.25[b]	156.25	141.00	110.50	144.00	10.10	162,523
$AVA_{1–35}$	1403.07[a]	119.47	130.00	154.07	158.93	8.70	175,569
SEM	54.56	7.82	5.92	8.43	6.91	0.44	11,345
P value	0.0130	0.1000	0.0680	0.0910	0.6310	0.4960	0.9170
Age: 49d	Villi height (μm)	Upper villi width (μm)	Botton villi width (μm)	Crypth deep (μm)	Muscularis thickness (μm)	Ratio	Surface of villi (μm^2)
Control	1300.60[a,b]	160.80[a,b]	192.25[a,b]	156.00	194.60	8.56	210,137[a,b]
$AVA_{1–21}$	994.83[b]	148.00[B]	161.40[b]	141.83	216.17	7.02	146,734[b]
$AVA_{1–35}$	1243.50[a,b]	197.86[A]	228.14[a]	132.38	187.43	9.00	268,105[a]
$AVA_{1–42}$	1039.67[b]	190.17[a,b]	215.17[a,b]	130.17	179.83	8.26	214,695[a,b]
$AVA_{1–49}$	1459.20[a]	170.40[a,b]	206.20[a,b]	147.20	198.50	9.10	274,864[a]
SEM	23.14	4.56	6.60	3.91	7.14	0.21	7390
P value	0.0170	0.0310	0.0220	0.5100	0.2310	0.3070	0.0110

[a,b]Means in a column without a common superscript are significantly different ($P < 0.05$).
[1]Control: basal diet without supplementation; $AVA_{1–21}$: 0.91 kg/t of Ava Cid P from 1 to 21 d; $AVA_{1–35}$: 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22–35 d; $AVA_{1–42}$: 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22 to 42 d; $AVA_{1–49}$: 0.91 kg/t of Ava Cid P from 1 to 21 d, 0.45 kg/t from 22 to 35 d and 0.23 kg/t from 36 to 49 d.
[2]Pooled SEM (standard error of the means).

Table 5. Effect of the Treatments on the Ileum Morphometric Analysis.

Age: 9d	Villi height (μm)	Upper villi width (μm)	Botton villi width (μm)	Crypth deep (μm)	Muscularis thickness (μm)	Ratio	Surface of villi (μm^2)
Control[1]	424.71	91.56[b]	102.29[b]	93.77	93.90	4.92	41,410[b]
$AVA_{1–21}$	473.08	116.02[a]	142.45[a]	91.80	102.09	5.32	62,663[a]
SEM[2]	10.41	4.47	5.58	3.64	3.90	0.19	3438
P value	0.0660	0.0380	0.0040	0.8450	0.4320	0.4460	0.0170
Age: 35d	Villi height (μm)	Upper villi width (μm)	Botton villi width (μm)	Crypth deep (μm)	Muscularis thickness (μm)	Ratio	Surface of villi (μm^2)
Control	940.89	131.80	144.02	111.74	136.92	8.36	133,045
$AVA_{1–21}$	872.47	103.24	124.90	110.11	154.47	8.19	105,637
$AVA_{1–35}$	972.79	120.43	123.67	131.44	137.35	7.28	116,025
SEM	24.43	4.14	4.92	4.18	6.77	0.26	5669
P value	0.2860	0.1420	0.3510	0.0630	0.6240	0.1890	0.3790
Age: 49d	Villi height (μm)	Upper villi width (μm)	Botton villi width (μm)	Crypth deep (μm)	Muscularis thickness (μm)	Ratio	Surface of villi (μm^2)
Control	741.83	147.55	183.29	134.33	233.46	6.27	139,845
$AVA_{1–21}$	777.51	169.04	202.33	128.43	201.97	6.10	144,028
$AVA_{1–35}$	814.48	151.76	218.89	138.06	199.15	6.21	158,357
$AVA_{1–42}$	852.04	164.80	218.91	141.92	221.48	6.01	164,718
$AVA_{1–49}$	874.09	181.27	211.98	126.69	217.52	6.68	166,832
SEM	23.14	4.56	6.60	3.91	7.14	0.21	7390
P value	0.4210	0.1370	0.4130	0.7320	0.5560	0.8960	0.7300

[a,b]Means in a column without a common superscript are significantly different ($P < 0.05$).

[1]Control: basal diet without supplementation; $AVA_{1–21}$: 0.91 kg/t of Ava Cid P from 1 to 21 d; $AVA_{1–35}$: 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22 to 35 d; $AVA_{1–42}$: 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22 to 42 d; $AVA_{1–49}$: 0.91 kg/t of Ava Cid P from 1 to 21 d, 0.45 kg/t from 22 to 35 d and 0.23 kg/t from 36 to 49 d.

[2]Pooled SEM (standard error of the means).

in the control group at 9 d ($P < 0.05$). At 35 d, the group $AVA_{1–35}$ had the highest villi height. At 49 d, there were conflicting results: birds from the $AVA_{1–49}$ group had higher villi than $AVA_{1–21}$ and $AVA_{1–42}$ birds and larger apparent villus surface area compared with $AVA_{1–21}$, but the treatments did not differ from the control group. At day 9, villus apical width, villus basal width, and apparent villus surface area of the ileum were higher with Ava Cid P inclusion ($P < 0.05$).

Several authors have indicated that butyric acid influences the intestinal morphometry of broilers [6, 19, 27]. Butyrate is recognized as an important respiratory fuel, effective source of energy for epithelial cell proliferation, and it is involved directly and/or indirectly in various mechanisms regulating cellular differentiation, growth, permeability, and gene expression [28]. Sodium butyrate is reportedly helpful in maintenance of intestinal villi structure after coccidial challenge [18]. In general, the increase in villus height and apparent villus surface area observed in the different segments of the small intestine of broilers may be attributed to a suppression of the growth of many pathogenic or non-pathogenic intestinal bacteria by the organic acids. Butyric acid reduces intestinal colonization and decreases inflammatory processes of the intestinal mucosa, which increase villus height. Thereby, secretion, digestion, and absorption of nutrients can be appropriately performed by the mucosa [29].

In conventional rearing and healthy chickens, as in this study, the benefits of sodium butyrate on small intestinal epithelia are minimal [8]. According to Shira et al. [30] and Panda et al. [27], butyrate supplementation is more helpful for young birds, when the intestinal and gut associated lymphoid tissue are developing and maturing, especially when there is no protection

Table 6. Effect of the Treatments on the Cytokines and MUC2 Expression.

Age: 21	IL1	IL10	MUC	TNF
Control[1]	0.999	1.000	1.000[a]	1.000[a]
$AVA_{1–21}$	0.989	0.992	0.984[b]	0.991[b]
SEM[2]	0.005	0.004	0.004	0.002
P value	0.2400	0.3120	0.0030	0.0411
Age: 35	IL1	IL10	MUC	TNF
Control	1.003	1.000	1.000	0.998
$AVA_{1–21}$	1.001	1.013	0.999	1.003
$AVA_{1–35}$	0.994	1.008	1.008	1.007
SEM	0.002	0.003	0.002	0.002
P value	0.1730	0.3144	0.0610	0.1140
Age: 49	IL1	IL10	MUC	TNF
Control	1.018	1.018	1.063	1.003
$AVA_{1–21}$	1.059	1.055	1.056	1.061
$AVA_{1–35}$	1.047	1.038	1.052	1.063
$AVA_{1–42}$	1.046	1.040	1.046	1.053
$AVA_{1–49}$	1.060	1.061	1.054	1.069
SEM	0.009	0.007	0.008	0.008
P value	0.4260	0.4042	0.1827	0.1620

[a,b]Means in a column without a common superscript are significantly different ($P < 0.05$).

[1] Control: basal diet without supplementation; $AVA_{1–21}$: 0.91 kg/t of Ava Cid P from 1 to 21 d; $AVA_{1–35}$: 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22 to 35 d; $AVA_{1–42}$: 0.91 kg/t of Ava Cid P from 1 to 21 d and 0.45 kg/t from 22 to 42 d; $AVA_{1–49}$: 0.91 kg/t of Ava Cid P from 1 to 21 d, 0.45 kg/t from 22 to 35 d and 0.23 kg/t from 36 to 49 d.

[2] Pooled SEM (standard error of the means).

from antibiotics. This can be inferred from our markedly different results at 9 and 35 d. Adil et al. [3], Ghazalah et al. [29], and Viola and Vieira [31] also found influences of organic acid blends on gut morphometry, including enhanced villus height in supplemented birds. However, in these studies, the organic acid supplementation levels were higher than the levels we used; their morphometry results were more consistent and associated with improvements in broiler performance.

Regarding the immune responses of the birds, the mRNA expression of MUC2 and TNF-α was lower in birds receiving butyrate than in the control group at 21 d (Table 6). At the other ages, and for the rest of the cytokines measured, no significant differences were found between treatments. The decrease in TNF-α was expected since butyric acid or its sodium salt can decrease pro-inflammatory cytokine expression and release via inhibition of nuclear factor-kappa B (NF-kB) activation [4, 32].

These results are similar to those found by Jiang et al. [6] and Zhang et al. [5], where diet supplementation with butyric acid in the first weeks also decreased the expression of the pro-inflammatory cytokines TNF-α and IL-6, with and without some health challenges. In vitro, Zhou et al. [33] found that butyrate by itself had no significant effect on mRNA expression of inflammatory cytokines but reduced the expression of IL-1β, IL-6, IFN-γ, and IL-10 in LPS-stimulated chicken macrophage cells.

It has been proposed that organic acids, especially butyric acid, reinforce the intestinal defense barrier by increasing the production of mucins and antimicrobial peptides. According to Willemsen et al. [34], short chain fatty acids enhanced the prostaglandin E_1/E_2 ratio secreted by subepithelial myofibroblasts in the intestine, which may support muco-protection by enhancing epithelial mucin expression. Furthermore, organic acids have anti-inflammatory properties [35, 36]. However, contrary to that evidence, our

results showed a decrease in MUC2. Gaudier et al. [37], who found that butyrate modulates MUC gene expression in human colonic goblet cells, found that this modulation varied according to the MUC gene. It is strongly dependent on the energy source provided to the cells and, in standard culture conditions, butyrate enhanced MUC3 and MUC5B, but not MUC2. They also suggest a different mechanism: that HDAC inhibition can be involved in the action of butyrate on MUC3 expression but not on MUC2, MUC5AC, and MUC5B gene expression.

CONCLUSIONS AND APPLICATIONS

1. The combination of substances that comprise Ava Cid P did not affect the performance of broiler chickens, but altered the mRNA expression of TNF-α and MUC2 in the early growth phase (1–21 d), and affected intestinal morphometry.
2. Further studies in birds with health or environmental challenges and other levels of product inclusion are still needed.

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