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The Effects of Direct-fed Microbial, Primalac®, or Salinomycin Supplementation on Intestinal Lactate Isomers and Cecal Volatile Fatty Acid Concentrations in Broilers[1]

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Abstract: Direct-Fed Microbials (DFM) are a putative alternative to the feeding of sub-therapeutic levels of antibiotics in the production of poultry and other livestock species. This study was designed to examine the effects of a commercial DFM (Primalac®), or salinomycin (SAL), a commonly used antibiotic and coccidiostat supplement, on fermentation patterns and lactate production in the cecum and the lower intestinal tract of broiler chickens. L-lactate and total lactate concentrations in the digesta fluid of the ileum decreased ($P<0.01$) with the DFM feeding in comparison to CON and SAL treatments while d-lactate concentration increased ($P<0.04$) in comparison to CON. Total cecal VFA concentration was lower ($P<0.003$) with DFM feeding and SAL than the CON. In the present study both dietary supplements, DFM and SAL, altered lactic acid and VFA concentrations in the cecum and intestines of experimental animals; however the full spectrum of mechanisms responsible for antibacterial properties and growth promotion associated with those changes remains to be elucidated.

Key words: Intestinal fermentation, volatile fatty acid, lactic acid, direct fed microbial

INTRODUCTION

Beneficial changes in intestinal and cecal fermentation patterns of volatile fatty acids (VFA) and lactate has been one of the modes of action hypothesized to explain the ability of direct-fed microbials (DFM) to control enteric pathogens. The role DFM play in the gastrointestinal fermentation of VFA and lactate is believed important in their ability to exclude pathogenic organisms. This is especially true of the cecum which is a major site of pathogen colonization in the broiler (Bjerrum *et al*., 2006). The increase in VFA and lactic acid concentrations causes the gastrointestinal pH to drop, preventing the colonization of acid sensitive pathogens (Nemcova, 1997; Zhang *et al*. 2003). In contrast, the mode of action of salinomycin is believed to lay in its ability to form lipid soluble complexes with certain cations and facilitate their transport across bacterial membranes (Spears, 1990). This would upset the intracellular electrolyte homeostasis of enteric pathogens causing increased energy expenditure thus impairing their ability to survive in the competitive microbial ecosystem of the gut. Salinomycin alters gastrointestinal fermentation and propionate in the forestomach of ruminants by increasing the production and molar percentage of the VFA propionate (Bagley *et al*., 1988; Spears, 1990). Salinomycin has also been shown to increase both the total concentration and molar percentage of propionate in *in vitro* cultures from chicken ceca (Marounek *et al*., 1996; Marounek *et al*., 1999). Little information is available on the effects of SAL on *in vivo* gastrointestinal fermentation in poultry.

This study was conducted to describe the effects of supplementation with the DFM, Primalac® ,or the ionophore, salinomycin, on changes in cecal microbial volatile fatty acids and intestinal and cecal lactate fermentation in broiler chicks.

MATERIALS AND METHODS

Experimental design: Fifty four, one-day old broiler chickens of mixed sex were fed a corn-soybean meal diet (17.08% CP, 2.4% fat, and 2830 kcal ME/kg). Chicks were assigned to one of following treatments: CON (no additives,), SAL (salinomycin, 50ppm of feed), and DFM (0.3%, Primalac®). DFM chickens were supplemented with a consortium of *L. Casei*, *L. Acidophilus*, *B. Thermophilium*, and *Enterococcus faecium* (Primalac®, Clarksdale, MO). Feed intakes and feed efficiency were as described by Chichlowski *et al*. (2007).

A completely randomized design was used. Chickens from each treatment were randomly assigned to different days for euthanasia followed by digesta sample collection. Individual bird measurements were the experimental units. Animal care and biosecurity were as previously described (Chichlowski *et al*., 2007). All experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at North Carolina State University.

Sample collection: On day 21, chickens were killed by cervical dislocation. Jejunal, ileal and cecal digesta samples were collected, frozen in liquid N_2 and stored at -20°C until preparation for analysis. Intestines between the bile duct and Meckel's diverticulum, and Meckel's diverticulum and the ileocecal junction were immediately ligated (30-60 seconds) after dissection and designated as the jejunum and ileum, respectively. Upon thawing, samples were immediately centrifuged at 13000 x g for 10 minutes to collect digesta fluid. Twenty µl of digesta fluid was mixed with 5 µl of 25% metaphosphoric acid. The samples were then stored at - 20°C until VFA and lactate analysis could be conducted. Because of the paucity of digesta available in the jejunum and ileum in these young chickens, VFA analysis was only conducted on cecal digesta fluid. d// Lactic acid analysis concentrations were estimated on samples of jejunal, ileal and cecal digesta fluid. In some cases, not enough digesta fluid was recovered to allow for analysis of VFA or dl/ lactic acid.

VFA and lactate analyses: Cecal digesta liquid concentrations of acetate, propionate, isobutyrate, butyrate, isovalerate and valerate were determined using a Schimadzu GC 15 gas-liquid chromatograph fitted with a 0.6' stainless column with SP 1200/ chromabsorb W packing. Injected volumes of digesta fluid were 2 µL for cecum, and 3 µL for ileum and jejunum. The flame ionization detector was set at 360°C. The analysis was conducted via programmed changes in column temperature. The column conditions for the cecum digesta were set initially at 130°C, and than increased by 5°C/min to 160°C. Temperature was then increased by 15°C/min to 190°C. Volatile fatty acid concentrations were calculated against an external standard using a computerized integrator (Shimadzu, Columbia, Maryland). Molar percentage of a single VFA was calculated by dividing the mM concentration of an individual VFA by the mM sum of all VFAs.

The concentration of l-lactate in the digesta fluid was determined with a l-lactate Analyzer (YSI 2300 Stat Plus, Yellow Springs, Ohio), whilst d-lactate concentrations were estimated using a colorimetric assay (Megazyme, Megazyme International, Ireland LTD Bray, CO).

Statistical analysis: The data were statistically analyzed using the ANOVA procedure of STATISTIX®8 (Analytical Software, Tallahassee, FL). Fisher's LSD test was used to test the significance of differences between the treatment means if overall significance was $P<0.05$. All statistical calculations on molar percentages of VFA were performed on *Arcsine* transformations of the data. Significant differences between means were accepted at $P<0.05$.

RESULTS

Table 1 lists the concentrations of cecal fluid VFA. Volatile fatty acid concentrations in the cecal digesta fluid reported in the present study were similar to that observed in broilers by Zhang *et al.* (2003) and Taylor (2002) in laying hens fed a wide variety of diets. Cecal fluid total VFA concentration was significantly reduced by the SAL and DFM diets by 42.9 and 30.8% (respectively) in comparison with the CON treatment ($P<0.01$; Table 1). Cecal acetate and butyrate concentrations decreased ($P\leq0.02$; Table 1) in the SAL and DFM chickens in comparison to the levels found in the CON chickens. No changes in the molar percentages of VFA in the cecal fluid were noted with SAL or DFM compared to CON.

Concentrations and molar percentages of both d and l isomers and total (dl) lactic acid in jejunal, ileal, and cecal fluid are presented in Table 2. Ileal and cecal l lactic acid concentrations (Table 2) were similar to that reported by Taylor (2002) in laying hens for a variety of diets whilst ileal concentrations were higher. d-Lactic acid concentrations were similar to the reported by Taylor (2002). Digesta fluid total lactic acid (l + d isomers) concentrations were 50-79% higher in the jejunum and ileum as compared to the cecum. Across treatments, the concentration of digesta fluid l-lactic acid was as much as 58% lower in the jejunum of DFM treated broilers as compared to CON and SAL treated broilers ($P=0.0001$; Table 2). d-Lactic acid concentrations were 218% higher ($P<0.0002$; Table 2) in the jejunal fluid of DFM chickens compared to CON and SAL treated chickens.

The only difference in the molar percentage of l or d digesta fluid lactic acid occurred in the jejunum where the percentage of l lactic acid molar percent decreased from a range of 94-96% in the CON and SAL chickens versus 82% ($P = 0.001$; Table 4) in the DFM treated chickens. The molar percentage of l lactic acid tended to be higher ($P<0.06$; Table 2) in the cecal fluid of DFM supplemented chickens as compared to SAL.

DISCUSSION

SAL and DFM decreased cecal acetate and butyrate concentrations which resulted in large decreases in total VFA (Table 1, 46% and 28% for SAL and DFM, respectively). VFA concentrations in the cecum can reflect the amount and type of feed intake by the broiler as well as the number and type of bacteria colonizing the cecum (Wang *et al.*, 2005). In the case of SAL, the decrease in total cecal VFA concentration may reflect a decrease in feed intake with SAL supplementation as noted in previous experiments in this laboratory (Chichlowski *et al.*, 2007), or they may represent an antibacterial effect of SAL (Engberg *et al.*, 2000). Although, no significant changes in the molar percentages of volatile fatty acids were noted with SAL or DFM, it is interesting to note the numerical decrease in the molar percentage of acetate/propionate with SAL. Similar trends were previously reported in ruminal fermentation using SAL diet (Bagley *et al.*, 1988; Spears, 1990).

Table 1: Cecal fluid VFA concentrations [mM/L] and molar percentages at 21 days of age[1]

	Diet[2]			
	CON	SAL	DFM	Significance
Concentration:	---------- mM/L ----------			
Acetate3	75.0 ± 7.2[a]	40.4 ± 6.1[b]	53.7 ± 7.0[b]	0.003
Propionate	8.0 ± 0.9	6.7 ± 0.8	6.3 ± 0.9	0.42
Isobutyrate	0.7 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.12
Butyrate	16.1 ± 2.0[a]	9.6 ± 1.7[b]	9.0 ± 1.9[b]	0.022
Isovalerate	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.30
Valerate	1.0 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.09
Total	101.2 ±9.1[a]	58.3 ± 7.7[b]	70.4 ± 8.7[b]	0.003
Molar:	---------- % ----------			
Acetate	60.2 ± 5.0	49.6 ± 4.2	57.0 ± 4.8	0.25
Propionate	16.0 ± 2.3	21.4 ± 2.0	18.7 ± 2.2	0.20
Isobutyrate	4.7 ± 0.5	5.8 ± 0.4	4.4 ± 0.5	0.09
Butyrate	22.7 ± 2.2	25.6 ± 1.9	21.4 ± 2.2	0.33
Isovalerate	3.6 ± 0.4	4.2 ± 0.6	3.4 ± 0.4	0.26
Valerate	5.6 ± 0.5	7.1 ± 0.5	5.7 ± 0.5	0.06
A/P ratio	3.9 ± 0.4	2.9 ± 0.3	3.4 ± 0.4	0.10

[a,b]Means in rows lacking a common superscript are significantly different (P≤0.05)
[1]n= 45. [2]CON = no additives, SAL = salinomycin (50 ppm), DFM = direct-fed microbial (Primalac®). [3]Least square means ± SEM

Table 2: Lactic acid isomer concentrations (mM/L) and molar percentages in intestines and cecum of 21 day-old broiler chickens[1]

	Diet[2]			
Parameter	CON	SAL	DFM	
l-lactic acid:	---------- mM/L ----------			Significance
Jejunum	11.0±1.4[a]	12.3±1.4[a]	4.6±1.2[b]	0.0001
Ileum	14.1±2.5	17.9±2.4	10.1±2.7	0.10
Cecum	1.0±0.1	0.9±0.1	1.2±0.1	0.25
d-lactic acid:	---------- mM/L ----------			
Jejunum	0.5±0.3[b]	0.8±0.4[b]	1.4±0.3[a]	0.0002
Ileum	0.5±0.4	1.4±0.5	1.3±0.5	0.31
Cecum	2.4±0.6	2.9±0.4	3.3±0.5	0.46
Total lactic acid:	---------- mM/L ----------			
Jejunum	12.0±2.4[a]	13.2±2.3[a]	4.8±2.4[b]	0.003
Ileum	14.4±2.6	20.0±2.7	10.9±3.3	0.10
Cecum	2.4±0.5	3.5±0.5	3.0±0.5	0.52
l-lactic acid	---------- % ----------			
Jejunum	96.3±7.0[a]	94.4±9.3[a]	82.2±7.0[b]	0.001
Ileum	97.4±0.9	95.9±0.9	97.3±1.2	0.45
Cecum	59.3±8.9	29.4±10.3	57.4±8.6	0.06
d-lactic acid	---------- % ----------			
Jejunum	4.2±3.8	5.6±4.7	23.0±4.0	0.108
Ileum	4.2±1.1	6.3±1.1	3.4±1.2	0.195
Cecum	67.7±5.2	77.8±4.9	68.1±4.9	0.286

[a,b]Means in rows lacking a common superscript are significantly different (P = 0.05). [1]n= 54.
[2]CON = no additives, SAL = salinomycin (50 ppm), DFM = Direct-fed microbial (Primalac®). [3]Least square means ± SEM

Unlike the SAL treated chickens, DFM supplemented chickens consumed similar amounts of feed as compared to CON (Chichlowski *et al*., 2007) but still had lower total cecal VFA concentration. Additionally, we noted no significant changes in molar percentages of VFA in DFM treated animals as compared to CON. This is similar to reports by Jin *et al*. (1998) and Mountzourism *et al*. (2007) demonstrating that supplementation of broilers with mixed *Lactobacillus* and other bacterial cultures did not increase cecal VFA concentration or change molar percentages. The lack of changes in molar percentages of VFA in the cecum of Primalac® supplemented broilers underscores the unique nature of individual DFMs to evoke different biological actions within the gastrointestinal tract while promoting growth and feed efficiency (Davis and Anderson, 2002). While others have reported similar results with other DFM as reported in the present study, there is still yet another body of literature that report increased concentrations of VFA with DFM supplementation (Patterson and Burkholder, 2003). Clearly the biological actions of all DFM are not the

same and broad generalizations about their potential mechanisms of action cannot be made.

Lactic acid concentrations, especially d lactic acid, are of general concern in the nutrition of ruminants and humans because they can contribute to systemic lactic acidosis. Taylor (2002) has expressed similar concerns about d lactic acid concentrations in the hindgut of the laying hen. Conversely, lactic acid production, in chickens fed DFM, likely inhibit pathogen colonization of the cecum and other parts of the intestinal tract. DFM significantly decreased ($P<0.0001$) l lactic acid concentrations in the ileum by 58% (Table 2) and increased ($P<0.0002$) jejunal d lactic acid by 68% as compared to CON. Total d/l lactic acid concentrations in the jejunum were decreased 60% with DFM compared to CON. This resulted in a decrease ($P<0.001$) in the molar percentage of l lactic acid with DFM compared to CON. No significant changes were noted in either lactic acid isomer concentrations or molar percentages in either the ileum or cecum. This lack of increase in lactic acid concentrations in DFM supplemented broilers has also been reported by Jin *et al*. (1998).

The biological significance of the shifts in jejunal lactic acid isomer concentrations in the jejunum with DFM is not clear. Different strains of *Lactobacilli* produce varying amounts of d and l isomers of lactic acid (Mirdomadi *et al*., 2002). It is possible that the jejunum provided an environment for a strain of bacteria that favored production of *d* lactic acid. This may account for the drop in *l* lactic concentration since both isomers are believed to be absorbed by the same H^+-linked monocarboxylate transporter (Garcia *et al*., 1994), thus allowing more competition for binding sites by d lactic acid. Alternatively, the drop in l lactic acid from the DFM treatment may indicate an increased total absorption of l lactic acid.

Here we report the effects of two commonly used poultry diet supplement, DFM and SAL, on intestinal lactate concentration as well as cecal lactate and VFA concentrations. Although both alter lactic acid and VFA concentrations in the cecum and intestines, respectively, the limited scale of this study makes it difficult to assign an association with these changes and the often reported growth promoting effects of both supplements on broiler growth and production.

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