Role of carvacrol and cinnamaldehyde in broiler cecal fermentations

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Aim of the study was to investigate the role of carvacrol and cinnamladehyde in modulating cecal microflora in broilers in an *in vitro* fermentation system. Cecal liquor was collected at the slaughter house, transported to the laboratory and immediately anaerobically diluted with a buffered solution; the so-obtained inoculum was dispensed in 10 ml syringes and 50 ml vessels (5 for each treatment) containing a pre-digested diet, carvacrol and cinnamaldehyde at the final concentration of 0, 75, 150, 300, 600, 900 e 1200 ppm. Syringes and vessels were sealed and incubated at 39°C for 24 h. Gas production was monitored from syringes throughout the study and after 24 hours ammonia concentration was measured from each vessel. A modified Gompertz bacterial growth model was used to fit gas production data as indicator of microbial growth. Curve fitting, total gas production, maximum rate of gas production, duration of the exponential phase and ammonia data were analyzed by ANOVA. Each syringe and vessel formed the experimental unit; the differences among means of groups were analyzed using the Student-Dunnet test. Differences with the control were considered statistically significant at $P< 0.05$. Data showed that, compared to control, carvacrol significantly depressed total gas production and exponential phase duration at 600, 900 and 1200 ppm (-53%, -64%, -76% and -62%, -73%, -72% respectively, $P<0.01$), while it increased the maximum rate of gas production at 600 and 900 ppm (+29%, +52%, $P<0.05$). Proteolysis, as indicated by ammonia concentration, was significantly reduced only by 1200 ppm of carvacrol (-26%, $P<0.05$). Cinnamaldehyde did not modify any intestinal microbial parameter. These results suggest that carvacrol can modulate the microbial fermentation when used at or above 600 g/ton of bioavaiable carvarcrol at cecal level. Cinnamaldehyde was not effective in modulating the broiler cecal flora.

Keywords: carvacrol; cinnamaldehyde; cecum; fermentation; broiler.

Introduction

Antibiotic growth promoters have played a significant role in animal production by improving growth performance and health status, but since their ban in the European Union, scientists are investigating risk-free alternatives. Any safe substance with potential capacity to modulate intestinal microbial activity is carefully considered. Essential oils (EO) from plant origin are under scrutiny and they are often constituted by very complex mixtures of compounds that can be very variable (Lee et al., 2004a). It has been long known that EO have antimicrobial and antioxidant properties, mainly due to the presence of phenolic groups (Valero and Salmeron, 2003, Shan et al., 2005, Ultee et al. 2002). Moreover, they seem to have other biological functions such as hypocholesterolemics (Craig, 1999) and stimulatory of pancreatic juice secretions resulting in improved digestion (Lee et al., 2004a). It is therefore of great interest to study the possible application of these compounds in poultry as an alternative to antibiotic growth promoters. Many *in vivo* studies about the use of EO, and in particular carvacrol and cinnamaldehyde, in broiler chickens production have been conducted (Lee et al., 2003b, 2004b) but there is no clear evidence that those compounds can positively enhance growth performance; furthermore it has been demonstrated that the addition of carvacrol at 200 ppm to a broiler diet lowered feed intake and weight gain when birds were fed the diet for 4 weeks, while it

improved the feed: gain ratio (Lee et al., 2003b). In this experiment we tried to investigate the role that carvacrol and cinnamaldehyde in an *in vitro* cecal fermentation system could have, in order to better understand the underlying mechanism in intestinal microflora modulation by these molecules.

Materials and methods

The study was conducted at the DIMORFIPA, University of Bologna, Italy.

A standard diet for broiler chickens (Table 1) was predigested *in vitro* to simulate ileal digestion as described by Malathi and Devegowda (2001). This was a stepwise procedure with an incubation of the ground feed (2 g; particle size < 1 mm) in 60 mL of pepsin solution (2,000 UI L^{-1}, HCl 0.1N; P7000 from porcine gastric mucosa; Sigma Chemical, St. Louis, MO, USA) in a shaking waterbath at 40°C for 45 minutes. In the second step 20 mL of a pancreatin-$NaHCO_3$ mixture solution (2 mg mL^{-1} pancreatin of 1M $NaHCO_3$; P1500, from porcine pancreas; Sigma Chemical, St. Louis, MO, USA) was added and all the mixture was reincubated for 2 h at 40°C to simulate the pancreatic phase.

After enzymatic digestion, the preparation was centrifuged (3,000 × g, 10 min., 4 C°), washed twice with distilled water, recentrifuged (3,000 × g, 5 min., 4 C°), and dried at 60°C overnight. Diet composition is reported in Table 1. Chemical analyses of the diet before and after predigestion are reported in Table 2. The predigested diet was used as the substrate in the *in vitro* fermentation study.

Within 20 min after slaughter, cecal content from broilers was removed and kept in a sealed nylon bag at 39°C during transfer to the laboratory. Cecal content was then diluted with buffer (ratio 1:5.5) and filtered through six layers of cheese cloth. The filtered liquid was used as inoculum. The buffer composition (McDougall 1948) was as follows: 9.8 g $NaHCO_3$ + 0.57 g KCl + 0.079 g $CaCl_2$•6H2O + 9.3 g Na_2HPO_4•12H_2O + 0.67 g NaCl + 0.12 g $MgSO_4$•7H_2O in 1 L of distilled water. Buffer pH was then adjusted to pH 6.7 by adding 3N HCl. The buffer solution was kept at 39°C and flushed with CO_2 for 20 minutes before use. The inoculum was dispensed into five 10 mL glass syringes (5 mL of inoculum in each syringe) and five 50 mL vessels (previously flushed with CO_2, 15 mL of inoculum in each vessel) per treatment, containing 20 and 100 mg of predigested diet, respectively (Piva et al. 1996). Syringes and vessels were sealed and incubated at 39°C for 24 h.

There were 13 treatments: control diet, or control diet added with carvacrol or cinnamaldehyde at 75, 150, 300, 600, 900 or 1200 ppm. Gas production was measured as described by Menke et al. (1979), using 10 mL glass syringes and recording the cumulative volume of gas produced every 30 min. Samples of fermentation fluid were collected from each vessel at time 0, 4, 8 and 24 h after incubation in a shaking water bath for ammonia analysis.

Chemical analyses of feed, fermentation fluid and intestinal contents

Analyses of the diets (crude protein, crude fiber, ether extract, ash and starch) were performed according to AOAC standard methods (AOAC, 2000; Method 954.01 for crude protein, Method 962.09 for crude fiber, Method 920.39 for ether extract, Method 942.05 for ash, Method 920.40 for starch) and Van Soest et al. (1991) for neutral detergent fiber (NDF) and acid detergent fiber (ADF) determinations. Gross energy of the predigested diet was measured by bomb calorimetry (model 1261; Parr Instrument, Moline, IL, USA).

Ammonia in fermentation fluid and intestinal chymus was measured as described by Searcy et al. (1967).

Statistical analyses

A modified Gompertz bacterial growth model (Zwietering et al. 1992) was used to fit gas production data. This model assumes that substrate levels limit growth in a logarithmic relationship (Schofield et al. 1994) as follows:

$$V = V_F \exp \{ - \exp [1 + (\mu_m e/V_F)(\lambda - t)]\}$$

where symbols have the meanings assigned by Zwietering et al. (1990): V = volume of gas produced at time t, t = fermentation time, V_F = maximum volume of gas produced, μ_m = maximum rate of gas production, which occurs at the point of inflection of the gas curve and λ = the lag time, as the time-axis intercept of a tangent line at the point of inflection.

The duration of the exponential phase was calculated from the parameters of the modified Gompertz equation, as suggested by Zwietering et al. (1992) with the following:

exponential phase (h) = $V_F/(\mu_m e)\{1 - \ln[(3 - \sqrt{5})/2]\}$.
Curve fitting was performed using the program GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). Total gas production, maximum rate of gas production, lag time, duration of the exponential phase and ammonia data were analyzed by ANOVA using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA) in a completely randomized design. Each syringe and vessel formed the experimental unit. The differences among means of groups were analyzed using the Student-Dunnet test. Differences were considered statistically significant at $P< 0.05$.

Results and discussion

The diet used for the experiment was a standard diet for starter broilers (0-21 d). Among the *in vitro* methods proposed by other researchers to simulate gastric and ileal digestion in broilers, we decided to use the one described by Malathi and Devegowda (2001) because of its likeness (shorter duration) with the *in vivo* process. Data relative to the diet composition and digestibility are shown in Table 1 and 2, respectively.

Table 1: Diet composition and chemical analysis (%, as-fed basis)

Ingredients	%	Nutrients	%
Soybean meal	40	Dry matter	87.38
Wheat meal	39	Crude proteins	23.02
Corn meal	13.90	Ether extract	4.94
Soybean oil	3.20	Crude fiber	3.68
Dicalcium phosphate	1.90	Ash	6.46
Calcium carbonate	0.60	Starch	34.85
Vitamin[1] and mineral Mix[2]	0.50	Lys	1.38
DL-Methionine 95%	0.30	Met+Cys	1.05
NaCl	0.24	Thr	0.89
L-Lysine HCl 98%	0.16	Calcium	0.97
Sodium bicarbonate	0.15	Phosphorus	0.74
L-Threonine	0.05	GE kcal/kg	4,002

[1]Supplied per kilogram of diet: vitamin A, 14,711 IU; vitamin D3, 4,404 IU; vitamin E, 16 IU; cyanocobalamin, 0.04 mg; riboflavin, 11 mg; niacin, 72 mg; d-pantothenic acid, 26 mg; choline, 1 g; menadione, 4.4 mg; folic acid, 1 mg; pyridoxine, 4.4 mg; thiamine, 2 mg; biotin, 0.2 mg.[2]Milligrams supplied per kilogram of diet: manganese, 131; zinc, 110; iron, 12; iodine, 2; copper, 12; selenium, 0.3.

Table 2: Chemical composition before and after *in vitro* digestion and diet digestibility

	Before digestion	After digestion	Digestibility (%)
Dry matter (g)	100.00	52.83	**47.17**
CP (% DM)	26.35	21.25	**78.75**
EE (% DM)	5.66	81.85	**18.15**
CF (% DM)	4.21	79.18	**20.82**
Ash (% DM)	7.39	15.37	**84.63**
Starch (% DM)	39.88	49.36	**50.64**

Gas production results and ammonia concentrations are shown in Tables 3 and 4, respectively. Compared with control, total gas production (V_F) decreased by high dosages of carvacrol. Significant lower gas production ranged from -53% when carvacrol was added at 600 ppm to -76% with carvacrol added at 1200 ppm ($P<0.01$). The same carvacrol levels lowered the duration of the exponential phase (log phase) from -62% at 600 ppm to -72% at 1200 ppm ($P<0.01$), while the maximum rate of gas production (μ_m) was significantly increased by carvacrol at 600 and 900 ppm (+29%, +52%, $P<0.05$). Cinnamaldehyde did not influence gas production kinetics.

Table 3: Gas production parameters from the *in vitro* fermentation of broiler cecal content with or without carvacrol or cinnamaldheyde. Values are means ± SD; n=5.

Dose (ppm)	V_F (mL)		μ_m (mL h^{-1})		Log phase (h)	
	Carvacrol	**Cinnamaldheyde**	**Carvacrol**	**Cinnamaldheyde**	**Carvacrol**	**Cinnamaldheyde**
0-ctr	6.65 ± 0.16	6.65 ± 0.92	0.21±0.01	0.21± 0.02	23.35 ± 1.23	23.35 ± 1.86
75	5.88 ± 0.35	6.63 ± 0.82	0.21±0.02	0.18±0.00	20.39 ± 0.63	27.18 ± 2.99
150	6.37 ± 0.71	6.52 ± 0.48	0.20±0.02	0.18±0.01	23.77 ± 1.29	27.01 ± 1.46
300	6.63 ± 0.18	5.79 ± 1.36	0.22±0.00	0.17±0.02	22.07 ± 0.75	24.42 ± 2.07
600	3.15 ± 0.53*	6.24 ± 0.43	0.27±0.03*	0.19±0.00	8.85 ± 2.69*	24.42 ± 1.49
900	2.36 ± 0.57*	7.04 ± 0.65	0.32±0.09*	0.19±0.02	6.31 ± 6.09*	27.32 ± 2.76
1200	1.62 ± 1.17*	6.84 ± 0.55	0.21±0.04	0.22±0.02	6.53 ± 6.48*	23.38 ± 2.46

*** within a column, indicates significant difference with control (P<0.05)**

Carvacrol significantly reduced ammonia concentration when used at 1200 ppm after 4, 8 , and 24 h of fermentation (-37%, -30%, and -26%, respectively; P<0.05), and when added at 900 ppm after 8 h (-18%; P<0.01). Cinnamaldehyde did not modify ammonia levels.

Table 3: Ammonia concentration (mmol L^{-1}) from the *in vitro* fermentation of broiler cecal content with or without carvacrol or cinnamaldheyde. Values are means ± SD; n=5.

Dose (ppm)	Ammonia at 4h		Ammonia at 8h		Ammonia at 24h	
	Carvacrol	**Cinnamaldheyde**	**Carvacrol**	**Cinnamaldheyde**	**Carvacrol**	**Cinnamaldheyde**
0-ctr	5.66 ± 0.67	5.66 ± 0.42	6.62 ± 0.45	6.62 ± 0.32	6.71 ± 1.14	6.71 ± 0.81
75	5.65 ± 0.62	5.09 ± 0.62	6.89 ± 0.67	6.60 ± 0.44	7.10 ± 0.77	5.83 ± 1.56
150	5.04 ± 0.14	5.05 ± 0.17	6.60 ± 0.64	6.81 ± 0.52	7.80 ± 1.11	6.04 ± 1.30
300	5.48 ± 0.38	5.59 ± 0.34	6.89 ± 0.67	6.67 ± 0.26	7.40 ± 0.31	4.92 ± 0.69
600	5.43 ± 0.94	5.95 ± 0.78	7.24 ± 0.37	6.50 ± 0.34	7.45 ± 0.71	6.00 ± 1.12
900	4.71 ± 0.98	5.41 ± 0.35	5.42 ± 0.40*	6.99 ± 0.34	5.42 ± 0.50	6.25 ± 1.61
1200	3.60 ± 0.39*	4.91 ± 0.36	4.63 ± 0.55*	6.65 ± 0.34	4.95 ± 0.58*	5. 55 ± 1.12

*** within a column, indicates significant difference with control (P<0.05)**

These data suggest that carvacrol can affect microbial fermentation at high concentrations by inhibiting the bacterial growth and metabolism even in the complex cecal ecosystem. This is justified by known carvacrol antimicrobial properties (Ultee et al.2002), that can, via its phenolic residue, disrupt the bacterial cell membrane and inhibit the replication. Other *in vitro* studies (Shanmugavelu et al., 2004) demonstrated that thyme oil, which contains thymol (21.38-60.15%) and carvacrol (1.15-3.04%) as main components (Asllani et al., 2003), included to a broiler cecal inocolum, significantly suppresses microbial fermentative capacity at 0.1%, 0.5%, 1%, 2% and 5% v/v. In an *in vivo* study Lee et al. (2004b) observed a significant weight reduction in animal fed a diet containing a blend of carvacrol (50%) and cinnamaldheyde (50%) at 200 ppm, while the group receiving a diet with carvacrol at 200 ppm alone maintained the same performance results of the control; this has been explained with a possible negative interaction between carvacrol and cinnamaldheyde.

In our study cinnamaldheyde was not effective in controlling intestinal fermentation. The possible *in vivo* use of carvacrol at the effective doses that we have observed *in vitro* should be subjected to the validation in broiler chickens of the proper efficacy and tollerance dose, and also of safety margin in case of accidental overdosing, as reccommended by EU guidelines for additive registion according to 1831/2003. Further investigations should be conducted about the opportunity to use multiple blends of those molecules to lower their inclusion rate in the diet to avoid the reduction of feed intake by birds.

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